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Hypoxia and xanthine oxidoreductase in the pathogenesis of abdominal aortic aneurysms

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HYPOXIA AND XANTHINE OXIDOREDUCTASE IN THE PATHOGENESIS OF ABDOMINAL AORTIC ANEURYSMS

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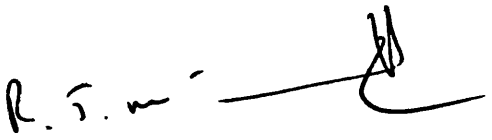

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DEDICATION

Catrina, Ellen and Roisin

Sorry it took so long!

It will all be worth it in the end!

GLOSSARY

AAA	Abdominal aortic aneurysm
ABC	Avidin biotin complex
AOD	atherosclerotic occlusive disease
ALP	Alkaline phosphatase
AMPS	Ammonium persulphate
APMA	p-aminophenylmercuric Acetate
AP-1	Activator protein-1
bp	Base pairs
Ca^{2+}	Calcium
ECL	Enhanced chemiluminescense
COX	Cyclooxygenase
C.pneumoniae	Chlamydia pneumoniae
DDW	Distilled deionised water
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DPI	Diphenyliodonium chloride
DEPC	Diethyl pyrocarbonate
DTT	Dl-Dithioreitol
DTPA	Diethylenetriaminepentaacetic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetracetic acid disodium salt
ELISA	Enzyme linked immunosorbent assay
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
Fe-S	Iron-Sulphur.
GAGs	Glycosaminoglycans
H+E	Haematoxylin and Eosin
H_2O	Water
HBSS	Hanks balanced salt solution
HCL	Hydrochloric acid
HIF	Hypoxia-Inducible factor
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
IFN-γ	Interferon–gamma

IgG	Immunoglobulin-G
IgA	immunoglobulin-A
IL-1β	Interleukin 1 beta
IL-2	Interleukin-2
IL-6	Interleukin-6
ILT	Laminated intraluminal thrombus
IMS	Industrial methylated spirits
IL-8	Interleukin 8
kDa	Kilodalton
KPO_4	Potassium phosphate
M	Molarity
mM	millimolar
MAP kinase	Mitogen-Activated Protein kinases
MCP-1	Monocyte chemoattractant protein -1
mg/dL	Milligram per decilitre
MMP	Metalloproteinase
Mo	Molybdenum
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane-type-1 matrix metalloproteinase
NAC	N-acetyl cysteine
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
$(NH_4)_2SO_4$	Ammonium sulphate
NFkB	Nuclear factor kappa B
3 NT	Nitrotyrosine
nm	Nanometers
NO	Nitric oxide
NO_3^-	Nitrate
NO_2^-	Nitrite
OD	Optical density
$O_2^{\bullet-}$	Superoxide
$OH^\bullet$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OPD	Orthophenylene diamine

PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PGE_2	Prostaglandin (E_2)
Pmoles	Pico ($x10^{-12}$) moles
PMSF	Phenylmethylsulphonyl fluoride
PO_2	Partial pressure of oxygen
PPE	Porcine pancreatic elastase
ROS	Reactive oxygen species
RONS	Reactive oxygen and nitrogen species
RNS	Reactive nitrogen species
rpm	Revolutions per minute
SAAANA	Succinyl trialanyl 4-nitroanilide
SDS	Sodium dodecyl sulphate (Lauryl Sulphate)
SOD	Superoxide dismutase
std	Standard deviation
S.E.M	Standard error of mean
TBE	Tris base, Borate, Ethylenediamine tetracetic acid disodium salt
TEMED	N, N, N', N'-Tetramethylethylene diamine
TIMPs	Tissue inhibitor of matrix metalloproteinases
TNBSA	2, 4, 6-trinitrobenzene sulfonic acid
t-PA	Tissue-type plasminogen activator
TNFα	Tumour necrosis factor-alpha
Tris-HCL	Tris[hydroxymethyl] aminomethane hydrochloride
Tween 20	Poloxyethylene sorbitan monolaurate
w/v	Weight per volume
µl	Microlitre
µg	Micrograms
u-PA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
v/v	Volume per volume
XDH	Xanthine dehydrogenase

XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

ABSTRACT

Aneurysmal degeneration is the end result of a multi-factorial process leading to the destruction of aortic wall connective tissue. Compelling evidence indicates that elastin and collagen destruction associated with increased production of enzymes capable of degrading these fibrillar extracellular matrix proteins forms the basis of this pathological process.

Xanthine Oxidoreductase (XOR) has been attributed roles in diverse pathologies primarily because of its capacity to generate reactive oxygen and nitrogen species (RONS). Evidence that hypoxia exists in arterial walls and that XOR is transcriptionally induced and activated by hypoxia leading to increased RONS, give the enzyme importance as a potential inducer of vascular pathology, as both hypoxia and RONS are known to activate transcription factors such as hypoxia-inducible factor (HIF-1), activator protein (AP-1) and nuclear factor kappa B (NFκB). I hypothesised that local hypoxia or RONS generated from XOR could modulate such transcription factors and increase the expression of the elastolytic enzymes MMP-2 and MMP-9, or they could directly affect MMP function resulting in enhanced elastolytic activity, a feature of AAA formation.

The level, localisation characteristics and activity of XOR in aortic tissue were assessed. It was found that no significant difference existed in XOR protein levels between AAA and non-AAA control tissue.

The effect of hypoxia on the expression and protein levels of MMP-2 and MMP-9 in cultured vascular smooth muscle cells (VSMCs) were assessed. In addition the effect of hypoxia on elastolytic activity of conditioned media from VSMC cultures was assessed. These experiments demonstrated that hypoxia did not influence MMP expression, protein levels or elastolytic activity in VSMC cultures.

Finally, aortic tissue was assessed for evidence of increased oxidant stress by measuring peroxynitrite levels and superoxide generating capacity. In addition, the effects of oxidant stress on MMP activation were assessed *in vitro*. These studies demonstrated that aortic tissue from AAA patients had evidence of increased peroxynitrite formation and oxidant stress activated MMPs.

INTRODUCTION

Chapter 1 Clinical aspects of abdominal aortic aneurysms

1.1 Definition

The term aneurysm is derived from the Greek word *aneurynein,* meaning to widen or dilate. A consensus definition of aneurysm published by the Society of Vascular Surgery and the International Society for Cardiovascular Surgery defined an abdominal aortic aneurysm (AAA) as a permanent localised dilation of an artery having at least a 50% increase in diameter compared with the expected normal diameter of the artery, or of the diameter of the segment proximal to the dilatation (*Johnston et al. 1991*). Based on a maximum diameter of 2.1 cm of the infrarenal aorta in healthy individuals, an AAA is present when the diameter exceeds 3.0cm.

1.2 Epidemiology

1.2.1 Prevalence

Estimates of the prevalence of AAA obtained from population screening surveys and autopsy studies vary between 1.3% and 8.4%, depending on the age group screened and criteria used for the definition of AAA (**Table 1.1** and **Table 1.2**).

Aneurysmal disease is predominately a condition of elderly men, being up to six times more common in men than women in population screening studies (*Scott et al. 1995*).

1.2.2 Incidence

The reported incidence of asymptomatic AAA varies between 3.0 and 117.2 per 100,000 person-years (*Wilmink et al. 1998b*) (**Table 1.3**). The age-adjusted incidence of AAA is rising and this increase appears to be real and not just the result of an increasing elderly population or improvements in diagnostic techniques. A nationwide study from Denmark reported an increase in the incidence of asymptomatic lesions from 7.1 per 100,000 to 25.8 per 100,000 person years from 1977 to 1990 (*Eickhoff 1993*). This increase was constant over all age groups examined.

The incidence of ruptured AAA (RAAA) varies from 1 to 21 per 100,000 person years and again appears to be increasing (*Wilmink et al. 1998a*) (**Table 1.4**). One study found a sevenfold increase in RAAA over a 36-year period as well as an increase in the age standardised mortality rate from RAAA, indicating that the rise in incidence was not caused by changes in age distribution of the population (*Drott et al. 1992*).

Location	Sex	Age	Numbers	Criteria (mm)	Prevalence (%)
Oxford (*Collin et al. 1988*)	M	65-74	426	5mm >SA	5.4
Oxford (*Collin et al. 1988*)	M	65-74	426	>40	2.3
Gloucestershire (*Lucarotti et al. 1993*)	M	65	4232	>25	8.4
Gloucestershire (*Lucarotti et al. 1993*)	M	65	4232	>40	1.3
Birmingham (*Smith et al. 1993*)	M	65-75	2669	>29	8.4
Birmingham (*Smith et al. 1993*)	M	65-75	2669	>40	3.0
Chichester (*Scott et al. 1995*)	M	65-80	2342	>29	7.6
Chichester (*Scott et al. 1995*)	F	65-80	3052	>29	1.3

Table 1-1 Prevalence of AAA in screened populations

Location	No examined	Period	Prevalence in men (%)	Prevalence in females (%)
UK (*Turk 1965*)	1544	1963-1964	2.3	1.6
USA (*McFarlane 1991*)	5244	1950-1984	2.6	1.2
Sweden (*Bengtsson et al. 1992*)	45838	1958-1986	4.3	2.1

Table 1-2 Prevalence of abdominal aortic aneurysms in autopsy studies

		Incidence per 100,000 per year	
Location	Men	Women	Total
Western Australia (*Castleden et al. 1985*)	117.2	33.9	36.5
England and Wales (*Fowkes et al. 1989*)	11.3	3.0	-
Scotland (*Naylor et al. 1988*)	-	-	63.6
Netherlands (*Pleumeekers et al. 1994*)	37.6	5.5	

Table 1-3 Incidence of abdominal aortic aneurysm

Location	Period	Incidence at beginning (per 100,000 per year)	Incidence at end (per 100,000 per year)	Overall mortality rate (%)
Swansea (*Ingoldby et al. 1986*)	1974-1983	7	17	80
Worthing (*Mealy et al. 1988*)	1979-1988	9	18	89
East London (*Thomas et al. 1988*)	1981-1986	13	21	81
Swindon (*Budd et al. 1989*)	1982-1987	9	17	86
Gotenborg (*Drott et al. 1992*)	1952-1988	1	7	85

Table 1-4 Trends in the incidence of ruptured abdominal aortic aneurysms

1.3 Risk factors

Risk factors for AAA formation include; increasing age, male gender, family history, previous vascular disease, hypertension, smoking and hypercholesterolaemia (*Wilmink et al. 1998a*). Several screening studies have shown that the prevalence of AAA increases with age (*Bengtsson et al. 1992; Morris et al. 1994*). Familial clustering of AAA was described by Clifton in 1977 (*Clifton 1977*). Powell and Greenhalgh interviewed 60 patients with AAA and identified that one third had at least one first degree relative with an AAA. The incidence among the patients' siblings was 7.3% and among their parents it was 8.6%. Mathematical analysis of AAA clustering in this study suggested that the inheritance pattern was multifactorial (*Powell et al. 1987*).
One study reported a tenfold increased risk of developing the condition in men with a first degree relative with an AAA, whereas others have found a lower relative risk (*Baird et al. 1995; Verloes et al. 1995*). The magnitude of the increased risk in first-degree relatives suggests a genetic component, although the influence of a common lifestyle cannot be excluded. Wilmink and Quick performed a pooled relative risk estimate of several risk factors for AAA from population based screening surveys and concluded that the risk factors most strongly associated with AAA were male sex and smoking. Patients with peripheral vascular disease and cardiovascular disease were twice as likely to have an AAA than those without these diseases, and hypertension was associated with a mildly increased risk of AAA. Diabetes and hypercholesterolaemia were not associated with increased risk in the population-based studies that considered these risk factors (*Wilmink et al. 1998a*).

1.4 Natural History and prognosis

The life expectancy of a patient with an AAA is lower than that compared to age matched controls, with the majority of deaths being due to coronary heart disease or aneurysm rupture (*Szilagyi et al. 1972; Conway et al. 2001*). The prognosis for non-operated AAA is poor with up to 40 % of the patients dying from rupture and three quarters of these deaths occurring within 2 years of diagnosis (*Szilagyi et al. 1972*). The 5-year survival of patients with aneurysms greater than 5 cm diameter not treated by surgery was 20% (*Szilagyi et al. 1972*). Similar data from Conway documenting the outcome of patients with AAAs larger than 5.5 cm in diameter who were turned down for elective open repair showed an overall 3-year survival rate of 17%. Patients with AAAs larger than 7.0 cm lived a median of 9 months. A ruptured aneurysm was certified as a cause of death in 36% of patients with an AAA of 5.5 cm to 5.9 cm, 50%

of patients with an AAA 6 cm to 7.0 cm and 55% of patients with an AAA larger than 7.0 cm (*Conway et al. 2001*).

Ruptured AAA (RAAA) is responsible for 10,000 deaths annually in the UK, accounting for 1.36 per cent of deaths in men and 0.45 per cent of deaths in women over the age of 65 in England and Wales (Office for National Statistics 1995). Operative mortality for RAAA is around 50% but fewer than half of those who rupture reach hospital alive (*Ingoldby et al. 1986*). Overall mortality is approximately 80-90% for all RAAA (*Ingoldby et al. 1986; Bengtsson et al. 1993; Katz et al. 1994*).

1.5 Risk of rupture

The risk of rupture of an AAA depends mainly on the maximum diameter of the AAA, however, it is also independently and significantly associated with female gender, current smoking and higher mean blood pressure (*Powell et al. 2001*). The UK Small Aneurysm study and North American Aneurysm detection and Management study (ADAM study) revealed than AAAs less than 5.5 cm had a 1%/year rupture rate (*UK Small Aneurysm Trial Participants 1998; Lederle et al. 2002*) The reported rupture rate for 6 cm and 7 cm AAAs have been quoted as 5% and 65% respectively (*Szilagyi et al. 1966; Sterpetti et al. 1991*).

Aneurysm enlargement/expansion, estimated to be exponential with a growth rate of approximately10% per annum has been shown to be related to the risk of rupture (*Bengtsson et al. 1993*). Expansion rates will vary between different patients and within the same patient over different time periods, however, AAAs observed to expand rapidly, i.e. > 5 mm in 6 months are regarded as being at high risk for rupture (*Bengtsson et al. 1993*).

1.6 Treatment

The aim of prophylactic elective repair of an AAA is to prevent death from AAA rupture and is indicated when the risk of death/major morbidity from the operation is less than the risk of death from rupture. Setting the intervention criterion too high risks losing lives owing to rupture occurring at small size, while setting the intervention criterion too low risks losing lives because of the mortality rate associated with elective repair in those whose AAA might never rupture.

Mortality rates of between 1% and 5% are published for elective aneurysm repair (*Hollier et al. 1992; Akkersdijk et al. 1994; Hak et al. 1996*). These published series undoubtedly contain significant publication and selection bias thus producing a falsely

low impression of surgical mortality. Others believe that the true mortality is 8% (*Blankensteijn et al. 1998*).

Estimation of the risk of rupture for an individual patient is difficult, but in general the principle determinant of rupture is AAA diameter. An aortic diameter greater than 5.5-6.0 cm is believed to be the level when the risk of surgery is less then the potential risk of rupture. Smaller AAAs (< 5 cm) rarly rupture, thus given an elective mortality of 5-8% expectant management of such AAAs appears to be pragmatic (*UK Small Aneurysm Trial Participants 1998; Lederle et al. 2002*). The UK Small Aneurysm Study, randomised 1090 patients, aged 60-76 years and fit for surgery, with asymptomatic aneurysms 4.0-5.5 cm to early surgery or ultrasound surveillance until the aneurysm either grew to 5.5 cm or became symptomatic when operation was undertaken. At a mean follow up of 4.6 years and analysed on an intension to treat basis, there were no statistical differences in mortality between the two groups. Cost analysis indicated that surveillance was a cheaper option than early surgery. The conclusion from this study was that AAAs less than 5.5 cm should be managed by ultrasound surveillance (*UK Small Aneurysm Trial Participants 1998*). A similar conclusion was found in the North American ADAM study (*Lederle et al. 2002*).

AAA repair is known to prolong life and five and ten year survival rates of 60 % and 40 % respectively have been reported (*Hollier et al. 1984; Stonebridge et al. 1993; Aune et al. 1995; Feinglass et al. 1995; Batt et al. 1999*). Survival is better than patients not undergoing repair, and several studies have suggested that the survival rate is as good as age and sex matched control populations. However, others suggest that it is below that of aged-matched controls, due to a higher incidence of coronary artery disease in the AAA population (*Hollier et al. 1984; Reigel et al. 1987; Johnston 1994; Aune et al. 1995; Batt et al. 1999; Yasuhara et al. 1999*). A Norwegian study found that the 10-year survival rate for all patients after AAA repair was 38% compared with an expected 52% (*Aune et al. 1995*). The standardized mortality rate was 1.30 indicating a 30% higher mortality rate than in a demographically matched population. A Canadian study came to similar conclusions, showing that the late survival rate of patients with AAA is significantly less than that of age and sex matched normal populations. Late deaths from cardiac and cerebrovascular causes were the most common causes of death (*Johnston 1994*).

Several published series have documented that quality of life returns to normal within 3 months of elective surgery and even improves by 6 months compared to preoperative levels (*Hennessy et al. 1998; Perkins et al. 1998; Malina et al. 2000*). Indeed

assessment of quality of life in survivors of rupture AAA repair was excellent at 6 months, a possible argument for aggressive treatment of this condition (*Hennessy et al. 1998; Bohmer et al. 1999*). There are no differences in the quality of life measurements after endovascular or open aneurysm repair (*Malina et al. 2000; Aquino et al. 2001*).

1.7 Screening

The difference in mortality rate from ruptured AAA and elective AAA repair, combined with the easy detectability of asymptomatic lesions, has led to calls for a national screening programme (*Harris 1992; Cheatle 1997*). A screening programme can be justified if two main criteria are met (*Wilmink et al. 1998a; Lindholt 2001*). Firstly, such a programme should be effective in reducing death or disability from a disease, and secondly, it should be cost effective. The recent Multicentre Aneurysm Screening Study (MASS study) has answered these two questions and supports data from previous pilot studies (*Scott et al. 1995; Wilmink et al. 1999; Heather et al. 2000; Lindholt et al. 2002; Scott 2002*).

The Gloucestershire community based screening service targets all males aged over 65 years (*O'Kelly et al. 1989; Lucarotti et al. 1993*). This population based screening approach has reported prevalence rates of 5% for aortas > 2.6 cm, 2.5 % for AAA > 3.0 cm and only 1% > 4.0 cm (*Crow et al. 2001*). The important conclusion is that 95% of men have a normal scan at age 65 and subsequent work has demonstrated that they can be safely excluded from further follow up as they are unlikely to develop a clinically significant AAA (*Emerton et al. 1994; Crow et al. 2001*).

The prevalence of AAAs and the incidence of rupture AAA is significantly less in females (*Scott et al. 1995; Choksy et al. 1999; Scott 2002*). In a controlled trial assessing the effects of screening in 9342 women aged 65-80 randomized to age-matched screen and control groups, the prevalence of AAA was six times lower in women than in men, and over 5- and 10-year follow-up intervals, the incidence of rupture was the same in the screened and control groups. The conclusion from this study was that screening women for AAA is neither clinically indicated nor economically viable (*Scott 2002*)

Chapter 2 Aortic structure and pathology of abdominal aortic aneurysms and formulation of hypothesis

Late onset degenerative aneurysms comprise 95% of all abdominal aortic aneurysms, and include the subgroups ' atherosclerotic aneurysm/non-specific aneurysm' and 'inflammatory aortic aneurysms'. The traditional view that atherosclerosis was the cause of AAAs was based on the histological association of atherosclerosis and AAA, as well as evidence from population epidemiological studies. However, over the last decade increasing advances in molecular biology have indicated that AAA is a unique and complex biochemical, structural, and physiological change in the constituents of the aortic wall. This chapter summarises the evidence from published literature.

2.1 Aortic wall structure and function

2.1.1 Structure

The aorta is a large elastic artery with a well developed tunica media consisting of elastin, collagen and vascular smooth muscle cells (VSMC) embedded in the extracellular matrix (ECM) connective tissue. The extracellular connective tissue matrix consists of proteoglycans, collagen, elastin, as well as smaller amounts of fibronectin, laminin and various plasma components. It provides structural support as well as serving multiple biological functions, including providing a medium for diffusion of nutrients and regulating cell migration and proliferation (*Ye et al. 1998*).

Proteoglycans possess one or more linear glycosaminoglycan chains attached to serine residues along a core protein. There are principally four types of proteoglycans (PG) present in the ECM, chondroitin sulphate PG, heparan sulphate PG, dermatan sulphate PG and keratan sulphate PG, all of which are synthesized by endothelial cells and smooth muscle cells. Proteoglycans play a role in extracellular architecture, arterial permeability, filtration, ion exchange and the regulation of cellular metabolism (*Ye et al. 1998*).

The elastin, collagen and VSMC are arranged in multiple concentric elastic lamella (*Clark et al. 1985; Dobrin 1988*). The radial sequence is elastin-cells-elastin-collagen bundles-elastin-cells-elastin-collagen bundles. The adventitia is organised in alternating lamellae of collagen and elastin (*Haas et al. 1991*).

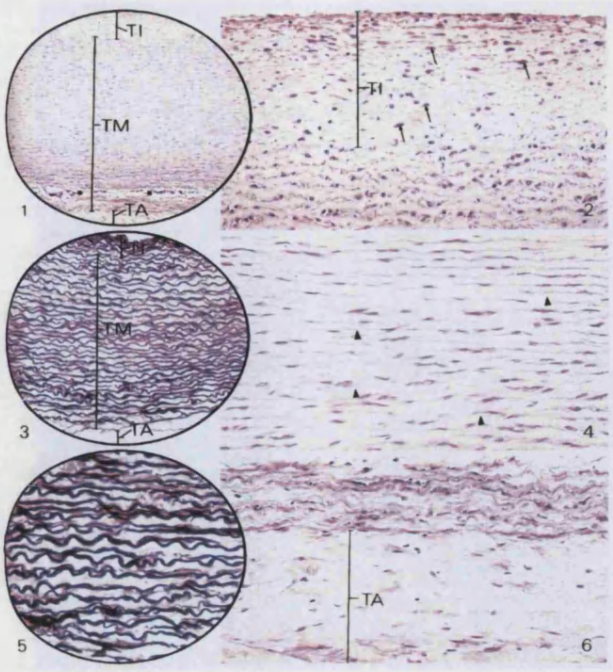

Figure 2-1 Aortic wall structure

Image 1(x65) is a longitudinal section through the entire thickness of the aorta demonstrating the tunica intima (TI), tunica media (TM) and tunica adventitia (TA). Image 2 shows the tunica intima at higher magnification (x 160) and consists of a lining of endothelial cells and connective tissue (collagen, elastin) and vascular smooth muscle cells (VSMCs)(arrows). Image 3 (x 65) and the higher magnification image 5 (x 160) stained for elastin (Van Gieson's stain) reveal the elastic laminae present in the aortic media (TM) as well as the deep portion of the intima (TI). Image 4 (H+E x160) shows the TM containing VSMCs arranged in a closely wound spiral between the elastic membranes. The arrowheads reveal the site of some of the lamellae, whose presence is recognized by the apparent absence of structure, which in turn is due to the absence of staining of elastic fibres. Image 6 (x60) demonstrates the outermost layer, the tunica adventitia (TA). The TA consists mostly of collagenous fibres that course in longitudinal spirals. There are no elastic laminae in the adventitia, but elastic fibres are present. The principle cell type is fibroblasts.

Elastin molecules are highly cross linked and assembled with microfibrils to form elastic fibres on the cell surface that are highly resistant to proteolytic degradation (*Mecham et al. 1995*). Elastin is a stable protein with a biological half-life of approximately 70 years and is produced by VSMC during early childhood, but is not synthesized in the adult aorta thus total elastin content is dependent on the degree of destruction (*Powell et al. 1992*).

Fibrillar collagen is composed of three polypeptide chains (α chains) wound around each other as a triple helical structure (*Ye et al. 1998*). Approximately 25 distinct α chains have been identified, which constitute about 15 types of collagen molecules. In the normal aortic wall the bulk of collagen exists in the form of two interstitial

collagens, type I and III in a ratio of 3:1 (*Menashi et al. 1987*). In addition types IV, V, VI are also found which account for 0.5-1% of the total arterial collagen content. It is synthesised continuously throughout life by VSMC within the media and by fibroblasts within the adventitia, and total collagen content reflects the net effect of synthesis and degradation within the aortic wall (*Ye et al. 1998*).

2.1.2 Function

The structural arrangement of the aorta imparts both extensile and tensile strength, allowing it to conteract haemodynamic force (*Clark et al. 1985; Dobrin 1988*). Elastin imparts viscoelastic properties to the artery. It is able to double its length before rapidly returning to its original dimensions, a property related to its structure of alternating hydrophilic and basic regions. The hydrophilic contain repetitive amino acid sequences that form beta turns that can readily be stretched. The basic regions are rich in lysine, which forms covalent cross-links by extracellular oxidation to allysine (*Ye et al. 1998*). Collagen has a tensile strength 20 times greater than that of elastin. It is very difficult to stretch, and can only extend a small proportion beyond its original length before structural damage occurs (*Dobrin 1988*).

Under normal physiological conditions elastin is the principle load-bearing element, with only 1% of collagen being loaded, resulting in an easily distensible vessel. As the load increases and the vessel stretches, collagen fibres uncoil and are progressively recruited as load bearing elements, resulting in a less distensible vessel. Collagen essentially acts as a strong indispensable ' safety net' (*Clark et al. 1985*).

2.2 Pathological changes in AAAs

2.2.1 Ageing

Epidemiological evidence suggests that the abdominal aorta gradually dilates with advancing age in a significant proportion of the population, but only a small proportion are prone to AAA formation (*Wilmink et al. 1998a; Wilmink et al. 2001*). The 'normal aorta' ageing is accompanied by elastin fibre calcification, elastin loss and increasing content of polar glycoproteins in the media (*Powell et al. 1992*). In youth the dry weight elastin content of the aortic wall is approximately 35% compared with about 25% at the age of 70 years and only 8-10% or less in an AAA wall (*Campa et al. 1987; Sakalihasan et al. 1993*). The effect is the recruitment of collagen at lower distending pressures and less aortic compliance (*Lanne et al. 1992; Sonesson et al. 1993*). These changes alter the mechanical properties of the aorta, such that the elasticity of the aorta in a 70-year-old patient is only a third of that of a 20 year old (*MacSweeney et al. 1992*).

2.2.2 Histological changes

Abdominal aortic aneurysms are characterised by extensive extracellular matrix remodelling with elastin destruction and tunica media disruption. The elastin volume fraction is significantly reduced and severely fragmented (*Campa et al. 1987; He et al. 1994*). In addition there is fragmentation of the internal elastic lamina, a reduction in VSMC numbers, thickening of the aortic intima and adventitia and a ubiquitous chronic inflammatory infiltrate (*Koch et al. 1990*).

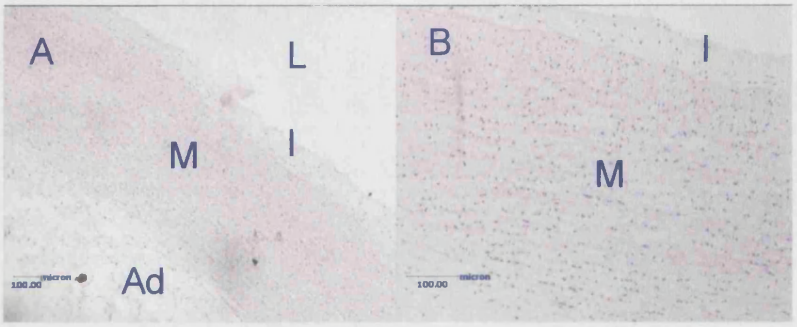

Figure 2-2 H+E stain of non-aneurysmal aortic wall

Image A (x 50) shows an intact intima (I), a thick well organised media (M) and adventitia (Ad). Image B (x100) demonstrates an organised media layer with concentric lamella units containing VSMCs.

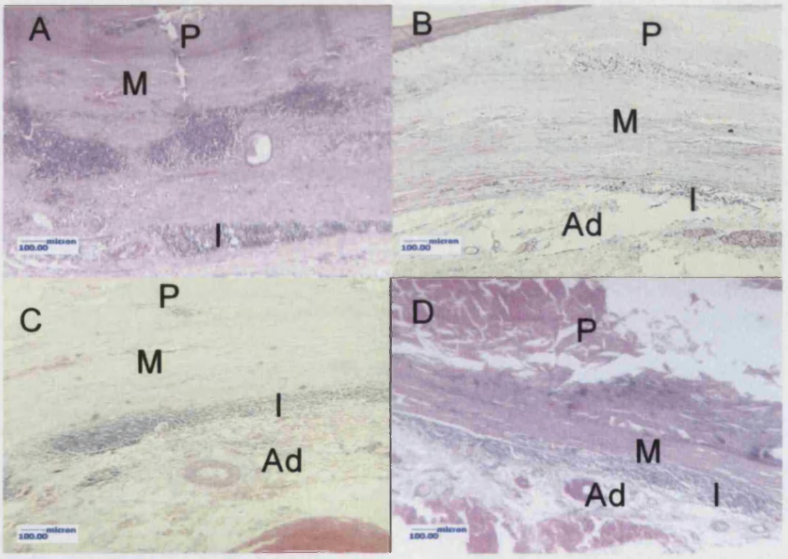

Figure 2-3 H+E stain of AAA wall

All sections show medial disruption and attenuation (M) with a varying degree of inflammatory infiltrate (I) situated within the media and at the medial-adventitial junction (M-Ad). P represents atherosclerotic plaques at the intima surface (x 50 magnification).

Inflammatory cells are seen in all AAA specimens and are usually confined to the media and adventitia. The subgroup 'Inflammatory aortic aneurysms' first described by Walker, which is characterised by an intense peri-aneurysmal inflammatory infiltrate is believed to be an extreme variant of the disease rather than a distinct entity (*Walker et al. 1972; Koch et al. 1990*).

The VSMC number and density in AAAs is decreased compared to controls. In aneurysm patients, the volume fraction of VSMC is only 2.2% compared to 22.6% in normals (*He et al. 1994; Lopez-Candales et al. 1997*). In addition VSMC apoptosis occurs within AAA tissue that is mediated by activated macrophages and T-lymphocytes within the AAA media and adventitia. (*Holmes et al. 1996; Lopez-Candales et al. 1997; Thompson et al. 1997; Henderson et al. 1999; Rowe et al. 2000; Jacob et al. 2001*). This is potential important as the VSMC population is the principal cell type responsible for the synthesis of all the components of the ECM.

2.2.3 Biochemical changes

Elastin loss is a marked feature of AAA formation. Quantitative analysis of aortic tissue reveals that elastin forms 35% of the dry weight of normal aortic wall but only 8% in AAA tissue (*Campa et al. 1987; Sakalihasan et al. 1993*). Histologically the remaining elastin is normal (*Tilson 1988*). There is no evidence of new elastin synthesis as levels of elastin mRNA are no different from normal aortic tissue (*Mesh et al. 1992*).

The elastase activity of AAA tissue has been shown to be raised and reciprocally related to elastin content (*Campa et al. 1987; Cohen et al. 1987; Cohen et al. 1988*). The extent of elastin destruction appears to be independent of aneurysm diameter, suggesting that elastin destruction occurs in the initial stages of the pathogenic process (*White et al. 1993*).

The total protein content of AAA tissue is 8.4 times greater than normal aortic tissue and 4.7 times greater than tissue from atherosclerotic occlusive disease (*Baxter et al. 1994*). He calculated that the volume fraction of collagen and ground substance is increased from 55 % to 95% in aneurysmal aortas as compared to controls (*He et al. 1994*). This is due to an increase in both the relative collagen concentration and absolute collagen content of AAA tissue (*Menashi et al. 1987; Rizzo et al. 1989*). However it is unclear whether this increase in collagen production is compensatory or pathogenic in abdominal aortic aneurysm formation, as stretch is known to be a stimulus for connective tissue formation (*White et al. 1993*). Collagen types I and III are increased but the ratio remains the same as in normal aortic tissue (*Menashi et al. 1987; Rizzo et al. 1989*). Increased collagenase activity has also been noted in AAA tissue which

supports the hypothesis that there is an increased turnover of collagen in AAA tissue, the increased synthesis being partly balanced by increased proteolysis (*Menashi et al. 1987; Vine et al. 1991*).

Interestingly the matrix abnormalities demonstrated in AAAs are not solely confined to the aneurysmal segment, but are also found in aortic tissue proximal to the aneurysmal segment. Baxter demonstrated a reduction in the elastin/collagen ratio throughout the arterial vasculature of AAA patients and Ward revealed that the mean diameters of all peripheral arteries were significantly greater in patients with aortic aneurysms suggesting that infrarenal aneurysmal disease may be a localised manifestation of a systemic dilating process (*Ward 1992; Baxter et al. 1994*). The increased turnover of proteins within the aortic media results in an imbalance of structural proteins and altered biophysical properties of the aorta, which explains the finding of decreased compliance which is a constant feature in AAAs (*MacSweeney et al. 1992; Sonesson et al. 1993*).

2.3 Potential Pathological mechanisms of AAA formation

2.3.1 Infra-renal aorta

Abdominal aortic aneurysms occur with greater frequency in the infrarenal segment of the abdominal aorta. The predilection for this site is probably multifactorial.

1. Firstly the number of elastic lamellae and therefore elastin content is markedly decreased in this segment compared with the thoracic aorta. As elasticity is directly related to the elastin content the aorta becomes stiffer and less compliant as it progresses distally (*MacSweeney et al. 1992*).
2. Secondly, the abdominal aorta does not have an abundant blood supply being relatively devoid of vasa-vasorum compared to the thoracic aorta (*Wolinsky et al. 1967*). Intimal atherosclerosis will further impair media nutrition and may predispose this site to nutritional insufficiency.
3. Atheroma is known to preferentially form in regions of low sheer stress, possibly as a result of prolonged contact between atherogenic factors and the vessel wall, a situation that exists in the aorta above the iliac bifurcation (*Moore et al. 1994*).
4. Flow dynamics are altered in the distal aorta above the iliac bifurcation. The pulse pressure within the aorta increases as the pressure wave passes distally due to aortic tapering, branching and decreased compliance (*Dobrin 1989*). At the iliac bifurcation the pressure wave is reflected, the amplitude of which depends on the ratio of the cross sectional area of the aorta and iliac vessels. The optimum ratio producing minimum reflection is 1:1.5, and in humans this ratio decreases from the ideal in

infancy to 0.75 by the age of 50 (*Gosling et al. 1971; Newman et al. 1973*). Theoretically, this ratio reduction will be accelerated by either atherosclerosis in the iliac system or aneurysm formation within the aorta, resulting in an increased magnitude of the reflected wave and localised areas of high pressure and a potential source of mechanical injury to the vessel wall. However MacSweeney found no evidence of localised high pressure zones in a small series in which direct measurements of pressure within an AAA showed it to be similar to that in the brachial artery (*MacSweeney et al. 1994*).

2.3.2 Genetics

Clifton was the first to report the increased prevalence of AAA in first-degree relatives and several other epidemiological studies have suggested a genetic cause or familial pattern for AAA formation (*Clifton 1977; Johansen et al. 1986; Bengtsson et al. 1992; Adams et al. 1993*).Two well-characterized genetic disorders have been associated with aortic fragility (Ehlers Danlos type IV syndrome) and aortic rupture (Marfans syndrome). These disorders are caused by mutations in the type III collagen gene (Chromosome 2) and the fibrillin gene (chromosome 15), respectively. However these congenital diseases represent distinct clinical entities that do not often manifest as true AAAs.

Studies investigating variations in the human elastin gene have failed to demonstrate any significant relationship to AAA formation (*Baxter et al. 1992; Gandhi et al. 1994*). A single base mutation of type III collagen has been described, but direct gene sequencing studies and population molecular genetics have failed to demonstrate that mutations or genetic variations in this gene are a common cause of AAA (*Kontusaari et al. 1990; Louwrens et al. 1993; Tromp et al. 1993; Anderson et al. 1996*). Powell identified a specific polymorphism in the type III collagen gene that was associated with increased aortic diameter and decreased compliance of the aorta and suggested that variations in the type III collagen gene may influence the mechanical properties of the ageing aorta and hence its susceptibility to disease and dilatation (*Powell et al. 1993*). In addition deficiency of α-1-antitrypsin (*Cohen et al. 1990; Elzouki et al. 1994*), the presence of the serum protein haptoglobin 2-1 phenotype (*Norrgard et al. 1984; Powell et al. 1990*) and the association between genetic variations of the Cholesterol Ester Transfer Protein (CETP) gene and AAA disease have all been investigated, however, none have been demonstrated to be responsible for significant numbers of AAAs (*Powell et al. 1990; Ramsbottom et al. 1994; Ramsbottom et al. 1997*). In conclusion, although familial clustering of AAA indicates a hereditary component the precise

genetic basis remains obscure. It seems likely that inheritance of AAA disease is multifactorial, involving a complex interaction between environmental influences and a genetic susceptibility (*Powell et al. 1987*).

2.3.3 Proteolysis

Elastin loss is critical and probably an initiating factor in the development of an AAA (*White et al. 1993*). Elastin is very resistant to both chemical and enzymatic degradation and only a few enzymes are known to degrade elastin, including leukocyte elastase (a serine protease) and the matrix metalloproteinases. Increased elastolytic activity in AAA tissue and plasma was first reported separately by Busuttil and Cannon in 1982 (*Busuttil et al. 1982; Cannon et al. 1982*). Initially believed to be due to leukocyte elastase (*Cannon et al. 1982*), Brown and Campa described an elastase that belonged to the matrix metalloproteinase (MMP) family rather than the serine proteinase (*Brown et al. 1985; Campa et al. 1987*). Subsequent work identified increased levels of elastolytic MMP-9 in homogenized AAA tissue compared to non-AAA tissue (*Vine et al. 1991; Newman et al. 1994b; Newman et al. 1994c*) .

2.3.4 Matrix metalloproteinases

2.3.4.1 Classification

The MMPs constitute a family of zinc-dependent endopeptidases. All MMPs share the following common features that allow their classification as a family (*Ye et al. 1998*).

1. They are all secreted as latent zymogens requiring activation for proteolytic activity,
2. They degrade extracellular matrix components,
3. They function at neutral pH,
4. They contain zinc at their active site and require calcium for stability, and
5. They are inhibited by specific tissue inhibitors of metalloproteinases (TIMPS).

The MMP family is divided into 4 main subgroups, mainly based on their substrate preferences (*Ye et al. 1998*):

1. **Interstitial collagenases**; that mainly degrade the interstitial collagen types I, II and III.
2. **Gelatinases;** which have a high affinity for gelatins (denatured collagen that has lost the typical helical structure), collagen type IV and elastin,
3. **Stromelysins**; that have a broad substrate specificity capable of degrading proteoglycans, type IV collagen, elastin and laminin, as well as activating interstitial procollagenases, progelatinases and other stromelysins and

4. **Membrane bound MMPs (MT-MMPs);** that appear to be able to cleave gelatins, type IV collagen and activate gelatinases.

Subgroup	Name	MMP Number	Substrate
Collagenases	Interstitial collagenase (Fibroblast-type collagenase)	MMP-1	Collagen types I, II, III, VI, X, gelatins, proteoglycans
	Neutrophil collagenase (PMN-type collagenase)	MMP-8	Same as MMP-1
	Collagenase-3	MMP-13	
Gelatinases	Gelatinase A (72 KDa type IV collagenase)	MMP-2	Gelatins, collagen types IV, V, X, XI, elastin, fibronectin, proteoglycans
	Gelatinase B (92 KDa type IV collagenase	MMP-9	Gelatins, collagen types IV, V, elastin, proteoglycans
Stromelysins	Stromelysin 1	MMP-3	Proteoglycans, fibronectin, laminin, elastin, gelatin, collagen types II, IV, V, IX, X
	Stromeylysin-2	MMP-10	As above
	Stromeylysin-3	MMP-11	Gelatin, fibronectin, proteoglycan
	Matrilysin	MMP-7	As above
	Metalloproteinase	MMP-12	Elastin
Membrane-type MMPs	MT-MMP-1	MMP-14	Collagen type IV, gelatin
	MT-MMP-2	MMP-15	

Table 2-1 Classification of matrix metalloproteinases

2.3.4.2 MMP protein structure

The collagenases and stromelysins consist of five domains, including a signal peptide, a propeptide containing a cysteine residue bonded to the active zinc site, which is lost on activation, a catalytic domain containing the zinc binding site, a so-called 'hinge region' that bridges the catalytic domain and the COOH-terminal domain, and a hemopexin or vitronectin like COOH-terminal domain that appears to be responsible for conferring substrate specificity (*Loftus et al. 2002*). The two gelatinases also have this five domain structure, however within the catalytic domain there are three fibronectin type II repeats which are believed to facilitate binding of gelatinases to their substrate(*Blankensteijn et al. 1998*). The MT-MMPs possess a unique transmembrane domain which anchors MT-MMPS to the cell surface (*Cao et al. 1995*).

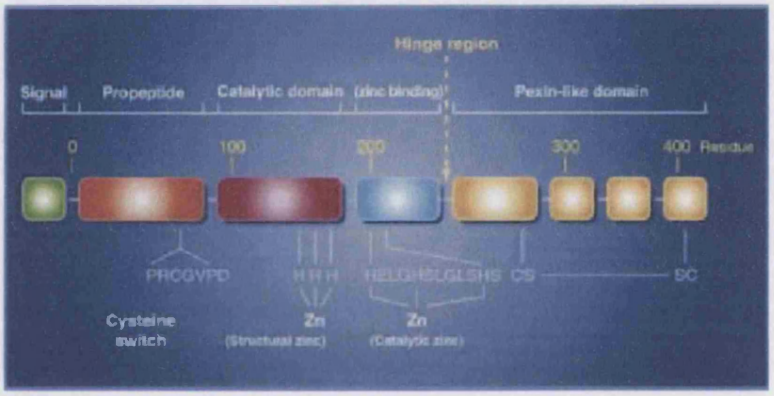

Figure 2-4 Structure of MMP protein

2.3.4.3 MMP activation

All MMPs are secreted as inactive zymogens that are then activated in the extracellular environment. The latency is due to the interaction between the cysteine thiol group within the propeptide and the zinc atom at the active site, which displaces the molecule necessary for catalysis (*Van Wart et al. 1990*). MMPs can be activated *in vitro* by physical, chemical (HOCL, mercurials, SDS) and enzymatic (trypsin, plasmin) treatments that separate the cysteine residue from the zinc atom (*Birkedal-Hansen 1995*). This critical step in the activation process, described as opening of the 'cysteine switch', is followed by a series of autocatalytic cleavages resulting in the complete excision of the propeptide and activation of the enzyme (*Van Wart et al. 1990; Woessner 1991; Loftus et al. 2002*).

The precise mechanism of *in vivo* activation is not fully resolved but plasmin and stromelysins 1 and 2 are believed to play a role (*Goldberg et al. 1990; Suzuki et al. 1990; Baricos et al. 1995*). Recent research has focused on the role of MT-MMP in the

activation of MMP-2 on the cell surface through the assembly of a ternary complex consisting of progelatinase A, TIMP-2 and activated MT-MMP (*Cao et al. 1995; Strongin et al. 1995; Sato et al. 1996; Okada et al. 1997*).

2.3.4.4 Tissue inhibitors of matrix metalloproteinases (TIMPs)

The natural activity of the MMPs is counteracted by the tissue inhibitors of matrix metalloproteinases, (TIMPs). All TIMPs act effectively against MMPs of the collagenase, stromelysin and gelatinase groups by forming inhibited, non-covalent 1:1 stoichiometric complexes with the MMPs and blocking access to their substrate (*Ye et al. 1998*). It is suggested that MMP-2 and MMP-9 (gelatinases) can form complexes with TIMPs in their zymogen form as well as active forms, whereas collagenase-type and stromelysin-type MMPs only form complexes after exposure of the active site (*Birkedal-Hansen 1995*).

TIMP-1 is synthesized and secreted by most connective tissue cells as well as by macrophages, and its expression is regulated by a variety of agents including growth factors (IL-1, TNF, and EGF). TIMP-2 frequently co-localised with MMP-2 and its expression is largely constitutive (*Ye et al. 1998*).

2.3.5 Physiological extracellular matrix remodelling

Extracellular matrix remodelling, involving the synthesis and degradation of matrix components, is a tightly controlled complex but normal physiological phenomenon involving complex cell-cell and cell-matrix interactions, the production of resident connective tissues, enzymes, activators, inhibitors and regulatory agents such as growth factors and cytokines (*Ye et al. 1998*). Disruption of these regulatory processes is thought to contribute to various pathological states such as aneurysm formation.

The key enzymes contributing to extracellular matrix turnover are endopeptidases from three major classes:

1. the metalloproteinases,
2. the serine proteases,
3. the lysosomal proteinases.

Matrix degradation initially takes place extracellularly at neutral pH and is catalysed by the metallo- and serine proteinases. Fragments are then phagocytosed and digested intracellularly. The metallo- and serine proteinase classes are linked in an activation cascade, with the metalloproteinases being downstream of the serine proteinases (**Fig 2.5**). In general, the serine enzymes have broader substrate specificity than the

metalloproteinases that tend to be more specialised. Under normal conditions, the lytic potential of these enzymes is held in check by their specific activators and inhibitors.

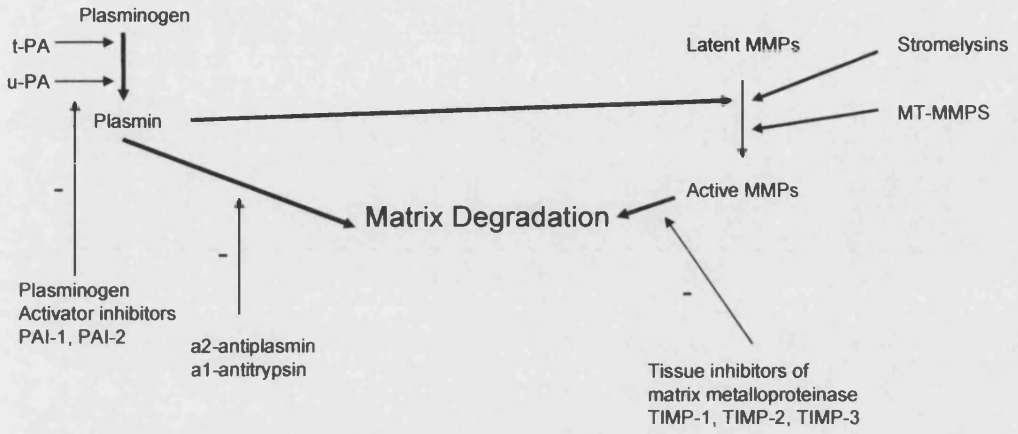

Figure 2-5 Extracellular matrix protease cascade

Abbreviations: t-PA: tissue-type plasminogen activator; u-PA: urokinase-type plasminogen activator; PAI: Plasminogen activator inhibitor; MMPs: Matrix metalloproteinases; MT-MMP: Membrane-type MMP; TIMP: tissue inhibitor of metalloproteinases. (*Ye et al. 1998*)

2.3.6 Pathological extracellular matrix remodelling and aneurysm formation

Several lines of evidence support the role of MMPs derived from both inflammatory cells and VSMCs in the initiation and expansion of AAA (*Shah 1997*). These include;

1. Evidence of an increase in net matrix-degrading activity in AAA tissue
2. Experimental studies showing that the infusion of elastolytic enzymes initiates the development of AAAs
3. Evidence of over-expression of MMPs in AAA tissue compared with tissue from atherosclerotic occlusive disease (AOD) or normal aortic wall
4. Evidence for reduced or unchanged expression of TIMPs
5. Increased expression of activators of pro-MMPs, such as plasmin and u-PA and t-PA in AAA.
6. Demonstration that inhibition of inflammatory cell recruitment or inhibition of MMP secretion and/or activity by cyclo-oxygenase (COX) inhibitors or by tetracycline derivatives decrease AAA development and expansion.

2.3.6.1 Evidence of increased elastolytic and collagenolytic activity in AAA tissue

Elastolytic and collagenolytic activity has been shown to be increased in AAA tissue compared to AOD tissue or normal controls (*Busuttil et al. 1980; Busuttil et al. 1982; Cohen et al. 1988; Cohen et al. 1989; Vine et al. 1991; Webster et al. 1991*).

2.3.6.2 Evidence from Experimental data

Animal experiments have shown that arterial infusion of elastase into animal aorta *in vivo* or human vessels *in vitro* is sufficient to cause aneurysmal dilatation. Following infusion the aortas show extensive elastin degradation. Interestingly such aneurysms do not rupture unless collagenase is also introduced. These experiments have given rise to the hypothesis that elastolysis leads to aortic dilatation and collagenolysis leads to aortic rupture (*Dobrin et al. 1984; Anidjar et al. 1990; Halpern et al. 1994*).

2.3.6.3 Over expression of MMPs in AAA tissue

Several MMPs have been identified and shown to be elevated in AAA tissue, including MMP-1(*Menashi et al. 1987; Vine et al. 1991; Irizarry et al. 1993; Newman et al. 1994c*), MMP-2 (*Freestone et al. 1995; McMillan et al. 1995; Patel et al. 1996a; Davis et al. 1998*), MMP-3 (*Newman et al. 1994d; Carrell et al. 2002*), MMP-9 (*Newman et al. 1994d; Sakalihasan et al. 1996a; Elmore et al. 1998*), MMP-12(*Curci et al. 1998; Annabi et al. 2002*) and MMP-13 (*Mao et al. 1999*).

2.3.6.3.1 MMP-2

Increased levels of MMP-2 mRNA have been demonstrated in AAA tissue compared to AOD or normal controls in several studies (*McMillan et al. 1995; Davis et al. 1998*), whereas other studies have shown no difference (*Tamarina et al. 1997; Elmore et al. 1998*). Pro-MMP-2 protein has been demonstrated to be elevated in AAA whole tissue homogenates compared to normal aortic tissue samples (*Freestone et al. 1995; Davis et al. 1998*) and VSMC and macrophages have been identified as the sources of MMP-2 in AAA tissue (*McMillan et al. 1995; Patel et al. 1996a; Davis et al. 1998; Crowther et al. 2000b*). In Davies study, MMP-2 was principally produced by mesenchymal cells, and its production was prominent when the mesenchymal cells were surrounded by inflammatory cells, suggesting paracrine modulation of MMP-2 expression from these cells (*Davis et al. 1998*).

Vascular smooth muscle cells derived from the aortic wall of AAA patients produce significantly more MMP-2 in culture than VSMC from normal or AOD patients (*Patel et al. 1996a*). Crowther demonstrated that VSMCs derived from AAAs produce 3-fold higher levels of MMP-2 than VSMCs derived from age-standardised atherosclerotic

controls. MMP-2 expression from other mesenchymal origin tissue (dermal fibroblasts) from patients with AAAs was similar to controls, suggesting that the increased expression of MMP-2 was specific to VSMCs. The increased MMP-2 production was a result of increased MMP-2 transcription, with comparable levels of TIMP-2 and MT-MMP-1 mRNA, suggesting that the regulation of MMP-2 gene expression was altered in aortic VSMCs from patients with AAAs (*Crowther et al. 2000b*). A second study from Goodall demonstrated that patients with AAAs have elevated VSMC MMP-2 levels in the vasculature, including venous tissue remote from the aorta. This again was shown to be due to increased MMP-2 expression from VSMCs within that tissue. This study supports the concept of widespread proteolysis in the vasculature of patients with AAA, suggesting that this may be due to over expression of MMP-2 from VSMCs (*Goodall et al. 2001*).

2.3.6.3.2 MMP-9

Increased levels of MMP-9 mRNA and pro- and activated MMP-9 have been shown to be raised in AAA tissue (*Vine et al. 1991; Newman et al. 1994c; Newman et al. 1994d; Freestone et al. 1995; McMillan et al. 1995; Sakalihasan et al. 1996a; McMillan et al. 1997; Tamarina et al. 1997; Elmore et al. 1998; Petersen et al. 2000; Yamashita et al. 2001*). Macrophages are believed to be the principal source of MMP-9 (*Newman et al. 1994b; McMillan et al. 1995; Thompson et al. 1995; Davis et al. 1998*), however, small levels of MMP-9 are detectable in normal aorta in the absence of inflammatory cells (*McMillan et al. 1995*) and VSMCs in culture from AAA patients do produce MMP-9 (*Patel et al. 1996a*), particularly in response to pro-inflammatory cytokines (*Galis et al. 1994*), which are known to be raised in AAA tissue (*Newman et al. 1994a; Szekanecz et al. 1994*). Recent evidence has demonstrated that MMP-9 levels are raised in the plasma of AAA patients, but the clinical use of this observation remains to be elucidated (*McMillan et al. 1999; Hovsepian et al. 2000*).

MMP-2 is believed to be the dominant elastolytic enzyme in the wall of small early aneurysms, with MMP-9 becoming most prominent as the inflammatory infiltrate increased in density (*Freestone et al. 1995; Thompson et al. 1995*). Freestone demonstrated that MMP-2 was the dominant elastolytic enzyme in smaller AAAs, whereas MMP-9 (produced from inflammatory cell) was the principle enzyme in larger AAAs (*Freestone et al. 1995*). Similar data from Petersen and McMillian suggests that MMP-2 levels correlate with increasing expansion of AAAs, but MMP-9 is required in larger AAAs for rupture, suggesting the role of MMP-2 in early AAA formation and expansion and MMP-9 in AAA rupture. (*McMillan et al. 1997; Petersen et al. 2000;*

Petersen et al. 2002). This has lead to the hypothesis MMP-2 drives aneurysm expansion by breaking down elastin, which via the formation of elastin degradation products attracts an inflammatory infiltrate that leads to further ECM degradation (*Crowther et al. 2000b*).

2.3.6.3.3 MMP-1

Experimental data suggests that for aneurysm rupture to occur aortic collagen must be destroyed (*Dobrin et al. 1984*). Increased collagenase activity has been described in AAA tissue by Busuttil and Manashi (*Busuttil et al. 1980; Menashi et al. 1987*). In the latter study, levels of collagenase activity in AAA tissue collected at elective repair were low whereas higher levels were noted in specimens from ruptured AAAs (*Menashi et al. 1987*). Increased levels of MMP-1 mRNA (*Tamarina et al. 1997*), and MMP-1 protein and activity have all been demonstrated in AAA tissue compared to normal or AOD tissue (*Irizarry et al. 1993; Newman et al. 1994c; Annabi et al. 2002*). The cellular sources of MMP-1 are macrophage and mesenchymal cells (*Irizarry et al. 1993; Newman et al. 1994b; Newman et al. 1994c*). Keen demonstrated that MMP-1 is transcriptionally upregulated in mesenchymal cells by inflammatory mediators such as platelet derived growth factor (PDGF) and interleukin 1β (*Keen et al. 1994*)

2.3.6.3.4 MMP-3

Stromelysin-1 (MMP-3) has a specific proteolytic action towards proteoglycans (*Palombo et al. 1999*). MMP-3 is present in increased levels in abdominal aortic aneurysms (*Newman et al. 1994d; Yoon et al. 1999*). Knox demonstrated that MMP-3 protein levels were increased in diseased but there was no difference between AAA and AOD (*Knox et al. 1997*). Carrell however, using reverse transcriptase and PCR showed that the expression of MMP-3 mRNA is significantly higher in AAA compared to AOD (*Carrell et al. 2002*). MMP-3 has been implemented in the destruction of the aortic media, both directly (*Woessner 1991*) and indirectly via the activation of pro-MMPs, namely MMP-1 and MMP-9 (*Suzuki et al. 1990*).

2.3.6.3.5 MMP-12

Human macrophage elastase (MMP-12) recovered from AAA tissue is seven fold greater than that from normal aorta (*Curci et al. 1998; Annabi et al. 2002*).

2.3.6.3.6 MMP-13

Collagenase-3 (MMP-13), a recently described matrix metalloproteinase with a limited tissue distribution and a highly regulated pattern of expression, has been shown to be elevated in AAA tissue (*Mao et al. 1999*). Mao demonstrated that MMP-13 mRNA was

present in AAA and AOD tissue but not in normal aortic control tissue. The expression of MMP-13 was 1.8-fold higher in AAA compared to AOD tissue. Immunoreactive MMP-13 was localized to medial VSMC which were also shown to express MMP-13 in culture (*Mao et al. 1999*).

2.3.6.4 Evidence of pro-MMP activators

MMPs are secreted as proenzymes that require activation. Evidence suggests that plasmin, MMP-3 and MT-MMP are all capable of activating MMPs *in vivo* (*Ye et al. 1998*).

2.3.6.4.1 Plasmin and Plasminogen activators

Plasmin is known to activate pro-MMPs (*Baricos et al. 1995*) and increased levels of plasmin have been shown in AAA tissue (*Jean-Claude et al. 1994*). In addition Reilly and Louwrens have shown that tissue plasminogen activators are elevated in AAA tissue and VSMC are a source of these activators (*Reilly et al. 1994; Louwrens et al. 1995; Reilly 1996*). The later study also demonstrated that there was no increase in the plasminogen activator inhibitors, (PAI-1 and PAI-2) in AAA tissue (*Louwrens et al. 1995*). Animal data suggests that blockage of plasminogen activators prevents the formation and rupture of experimental aneurysms in animals (*Allaire et al. 1998*).

2.3.6.4.2 Membrane type metalloproteinases (MT-MMPs)

Recent evidence suggests that MMP-2 can be activated on the cell surface through the assembly of a ternary complex consisting of pro-MMP-2, TIMP-2 and activated MT-MMP (*Cao et al. 1995; Strongin et al. 1995*). Crowther demonstrated that the MMP-2-TIMP-2-MT-MMP-1 enzyme system was localised to VSMC within the medial layer of the arterial wall, suggesting that this system may play an aetiological role in the pathogenesis of AAAs, however further work did not demonstrate an increased expression of MT-MMP-1 in AAA tissue compared to AOD tissue (*Crowther et al. 2000b, 2000a*), and levels of MT-MMP-1 were not elevated in vascular tissue remote from the aorta (*Goodall et al. 2001*). In contrast Nollendorfs demonstrated that MT-MMP-1 m RNA and protein were increased in AAA compared with AOD and normal tissue. Immunohistochemical analysis localized MT- MMP-1 to aortic VSMC and macrophages. AAA tissue demonstrated a greater capacity to activate exogenous pro-MMP-2 compared with atherosclerotic and normal aortic tissue suggesting that it may be important in AAA pathogenesis through its ability to activate pro-MMP-2 (*Nollendorfs et al. 2001*).

2.3.6.4.3 Prostaglandin E_2 (PGE_2)

The arachidonic acid metabolite PGE_2 has also been identified as an important regulator of the matrix metalloproteinases and has been shown to be elevated in AAA tissue compared to normal controls (*Holmes et al. 1997*). However, no difference in PGE_2 was observed between AAA tissue and AOD tissue (*Reilly et al. 1999*). Macrophages within the aortic wall inflammatory are the source of PGE_2 (*Holmes et al. 1997; Walton et al. 1999*). This study also demonstrated that its expression was controlled by the Cox-2 isoform of cyclo-oxygenase. Experimental data using Indomethacin, an inhibitor of cyclo-oxygenase, has demonstrated that decreased production of PGE_2 are associated with decreased AAA formation (*Holmes et al. 1996; Miralles et al. 1999; Walton et al. 1999*).

2.3.6.5 Tissue inhibitors of MMPs

Another possibility which may explain increased proteolytic activity in AAA would be a deficiency of the tissue inhibitors of metalloproteinases (TIMPs). In an animal model of AAA formation, local seeding of smooth muscle cells retrovirally transfected with TIMP-1 cDNA resulted in TIMP-1 local over expression, decreased MMP-9 and MMP-2 activity, elastin preservation and prevention of aneurysmal degeneration and rupture, suggesting that lack of TIMP-1 may be a reason for AAA development (*Allaire et al. 1998*).

Brophy demonstrated that levels of TIMP-1 are decreased in AAA tissue (*Brophy et al. 1991*), whereas other studies have shown no difference between AAA and normal tissue (*Annabi et al. 2002*), or any difference in TIMP-1 and TIMP-2 levels between AAAs of differing sizes (*Petersen et al. 2002*).

Elmore demonstrated that levels of TIMP-1 mRNA were not significantly different in AAA tissue compared to control or AOD tissue, but there was a small but statistically significant increase in TIMP-2 mRNA in AAA tissue (*Elmore et al. 1998*). Taminara showed that mRNA for both TIMP-1 and TIMP-2 were increased in AAAs, but the ratio of MMP to TIMP was significantly higher in AAA patients resulting in net elastolysis and matrix degradation (*Tamarina et al. 1997*). McMillian found no difference in TIMP-2 mRNA between AAA and AOD or normal aortas, but the level of MMP-2 mRNA was significantly increased resulting in a significantly higher MMP: TIMP ratio. TIMP-2 is constitutively expressed and co-localises with MMP-2 to VSMC and macrophages (*McMillan et al. 1995; Crowther et al. 2000b*).

2.3.6.6 Inhibition studies

Additional evidence to support the role of MMPs in the pathogenesis of AAAs is obtained from experimental animal data. Doxycline, tetracycline or chemical derivatives of these compounds have been shown to decrease AAA development in animal and human aortic explant models of aneurysm disease (*Petrinec et al. 1996a; Petrinec et al. 1996b; Boyle et al. 1998; Curci et al. 1998*). These drugs limit the disruption of medial elastin without altering the inflammatory response. They are believed to decrease the production of MMP-9 (*Petrinec et al. 1996b; Boyle et al. 1998*).

Early *in vivo* studies have shown that tetracycline penetrates the wall of the aorta in humans, decreasing MMP-9 and monocyte chemoattractant protein -1 (MCP-1) in a concentration dependent manner (*Franklin et al. 1999a, 1999b*). In addition Curci has shown that if Doxycline is administered to AAA patients preoperatively, both macrophage expression of MMP-9 and post-translational activation of pro-MMP-2 are decreased (*Curci et al. 2000*). In the only clinical study to-date Doxycline has been shown to decrease the expansion of small aneurysms (*Mosorin et al. 2001*). Similar results were obtained by synthetic MMP inhibitors in further animal studies (*Bigatel et al. 1999; Moore et al. 1999*).

Indomethacin, a cyclo-oxygenase type 2 (Cox-2) inhibitor has also been shown to decrease AAA formation in an animal model by decreasing macrophage expression of MMP-9 (*Holmes et al. 1996; Miralles et al. 1999*). Additional evidence shows that Indomethacin or mefanamic acid abolishes PGE_2 secretion and reduces IL-1β and interleukin-6 (IL-6) secretion in explants from AAA patients (*Franklin et al. 1999c; Franklin et al. 1999d; Walton et al. 1999*). These studies also report that the growth rate of AAAs is decreased if patients are treated with non-steroidal anti-inflammatory drugs.

Experimental data suggests that HMG-CoA reductase inhibitors (Statins) decrease MMP-9 secretion and activity in condition media from cultured human and mouse macrophages (*Bellosta et al. 1998*). Recent evidence also suggests that this group of compounds can decrease MMP-2 expression and activity and interleukin-6 (IL-6) levels in human AAA tissue explants(*Evans et al. 2002*). It is believed that these drugs work by reducing the transcription factor nuclear factor-kappa-B (NF-κB) expression, an intracellular oxidant dependent transcription factor that is required for cytokine up-regulation of MMP-1, MMP-3 and MMP-9 (*Bond et al. 2001*).

2.3.7 Inflammation

2.3.7.1 Histology

Abdominal aortic aneurysms are characterized by a non-specific ubiquitous chronic inflammatory infiltrate (*Koch et al. 1990; Brophy et al. 1991*). In approximately 10% of AAAs the inflammatory response is so intense as to be macroscopically detectable as the so-called ' inflammatory aneurysm ' which is characterized by a dense perianeurysmal fibrotic infiltrate, which may extend and involve adjacent organs (*Walker et al. 1972; Sterpetti et al. 1989*).

Immunohistochemical analysis identifies CD3+ T lymphocytes, macrophages and polyclonal B-lymphocytes. The infiltrating lymphocytes were often found in clusters, with B-cells surrounded by T cells often located around the adventitial vasa-vasorum, as well as associated scattered macrophages (*Koch et al. 1990*). Inflammation is a feature of both AAAs and aortic occlusive disease however the infiltrate differs in the following ways:

1. The lymphocytes present in aortic occlusive disease are predominately T- cells, while both T and B cells have been identified in AAA tissue
2. Adventitial inflammation is seen only in more advanced stages of aortic occlusive disease, while it is a consistent feature of AAA
3. The CD4+: CD8+ ratio is 7.6:1 in AAA compared to 2:1 in the peripheral circulation and 4.3:1 in aortic occlusive disease
4. Macrophages are distributed throughout the wall in AAA but in aortic occlusive disease they are limited to the plaque (*Beckman 1986; Koch et al. 1990 ; Brophy et al. 1991*).

Experimental animal data support the role of the inflammatory response in the etiology of AAA formation. Gertz and Anidjar induced aneurysm development in rabbit carotid and rat aorta by the injection of calcium chloride and elastase respectively and found that dilatation corresponded with the inflammatory infiltrate, which suggests that this forms a crucial central mechanism in the pathogenesis of AAAs (*Gertz et al. 1988; Anidjar et al. 1992*). Freestone demonstrated that the density of inflammatory cells within the aortic wall was the histological feature most clearly associated with AAA expansion and rupture (*Freestone et al. 1995*).

2.3.7.2 Function of inflammatory infiltrate

2.3.7.2.1 Cytokine production

Inflammatory cells secrete a variety of cytokines which activate and influence local mesenchymal cells (*Galis et al. 1994*). Tumour necrosis factor-alpha (TNF-α) (*Pearce et al. 1992; Newman et al. 1994a*), Interleukin 1β (IL-1β) (*Pearce et al. 1992; Newman et al. 1994a*), Interleukin-6 (IL-6) (*Szekanecz et al. 1994*), Interferon gamma (INF-γ) (*Szekanecz et al. 1994*), Monocyte chemoattractant protein (MCP) (*Koch et al. 1993; Szekanecz et al. 1994*) and Interleukin 8 (IL-8) (*Koch et al. 1993*) have all been shown to be increased in diseased aortic tissue. Plasma levels of IL-1β, IL-6, interleukin-6, TNF-α and INF-γ have been demonstrated to be increased in AAA patients compared to both patients with ischaemic heart disease or normal controls (*Juvonen et al. 1997*). Indeed, INF-γ concentrations were predictive of increased rates of expansion of AAAs (*Juvonen et al. 1997*).

Recent interest has focused on differential expression of cytokines between AAA and AOD. Reilly showed that AAA and AOD whole tissue organ culture secreted more IL-6 compared to normal tissue, however, AOD was associated with a much higher level of IL-6 expression than AAA (*Reilly et al. 1999*). Contradicting this observation is work from Shteinberg who demonstrated that AAA tissue samples contained significantly higher mean TNF-α and IL-6 levels compared to the AOD samples, but there was no differences related to IL-1β levels (*Shteinberg et al. 2000*). Davis, who analysed cytokine expression in tissue with an ELISA showed that AAA tissue contained higher levels of IL-10 compared to AOD tissue, whereas higher levels of the IL-1β, TNF-α and IL-6 were found in AOD (*Davis et al. 2001*).

The possible role of cytokines in AAA formation is demonstrated by evidence from experimental data. Indomethacin or mefanamic acid have been shown to abolishes PGE_2 secretion and reduces IL-1β and IL-6 secretion in explants from AAA patients as well as decrease the expansion of AAAs in a small cohort of patients (*Franklin et al. 1999c; Franklin et al. 1999d; Walton et al. 1999*). Hingorani showed in an animal model of AAA formation that blockage of TNF-α with a binding protein inhibited AAA formation (*Hingorani et al. 1998*).

The potential effects of these cytokines maybe to;

1. Increase angiogenesis and neovascularisation. TNFα is known to promote neovascularisation (*Szekanecz et al. 1994*) and medial neovascularisation is increased in AAA tissue compared to normal or AOD tissue (*Holmes et al. 1995*).

2. Attraction and recruitment of further inflammatory cells. TNFα and IL-1β incease the expression of intercellular adhesion molecules in cultured human aorta epithelial cells which are known to be elevated in AAA (*Davis et al. 1993; Szekanecz et al. 1994*). In addition IL-8 and Monocyte chemoattractant protein are known to be elevated in AAA tissue (*Koch et al. 1993; Szekanecz et al. 1994*).

3. Influence the expression of MMPs. Cytokines influence MMP gene expression at transcriptional level (*Mauviel 1993*). Keen demonstrated that AAA derived VSMC in culture increased MMP-1 mRNA expression when stimulated with IL-1β (*Keen et al. 1994*). Similar results were obtained by Galis who showed that VSMCs stimulated with IL-1β and TNF-α expressed several MMPs, including MMP-1, 2, 3 and 9. Interestingly mRNA and protein levels of TIMPs 1 and 2 appeared unaffected in the latter study, supporting the concept that cytokines alter the balance between matrix degradation and synthesis (*Galis et al. 1994*).

2.3.7.2.2 MMP production

Shapiro showed that as monocytes migrate into tissues and differentiate into macrophages, the gene expression for a variety of MMPs particularly, MMP-1, MMP-3 and MMP-9 dramatically increase (*Shapiro et al. 1990*). Subsequently the macrophage has been identified as a source of several MMPs in AAA tissue (*Irizarry et al. 1993; Newman et al. 1994b; McMillan et al. 1995; Thompson et al. 1995; Curci et al. 1998; Annabi et al. 2002*).

2.3.7.2.3 Production of MMP activators

Infiltrating macrophages have been demonstrated to be a source of PGE_2 within AAA tissue (*Holmes et al. 1997; Walton et al. 1999*).

2.3.8 Infection and aneurysm formation

As chronic inflammation is a contributing factor in the pathogenesis of abdominal aortic aneurysms, antigens such as *Chlamydia pneumoniae* (*C.pneumoniae*) have been suggested as possible triggers for the inflammatory response.

The association between *C.pneumoniae* and arterial disease is well recognised, but there is no proof that *C.pneumoniae* plays any clinically significant role (*Ong et al. 1996; Lindholt et al. 1999*). Immunohistochemical evidence and the demonstration of C. *pneumoniae* cDNA in AAA tissue suggests that C.pneumoniae may play a role in the pathogenesis of AAA formation (*Blasi et al. 1996; Juvonen et al. 1996; Ong et al. 1996; Juvonen et al. 1997; Petersen et al. 1998; Halme et al. 1999; Meijer et al. 1999*). In one study C. *pneumoniae* specific DNA was detected at a significantly higher

frequency in the wall of AAA samples compared to tissue from normal aortas (*Petersen et al. 1998*).

Antibodies to C. *pneumoniae* can be detected in between 40-80% of AAA patients (*Blasi et al. 1996; Lindholt et al. 1999; Lindholt 2001*). Meijer demonstrated the presence of C. *pneumoniae* antigens in the absence of specific DNA in abdominal aortic aneurysms, suggesting persistence of the antigens rather than a persistent infection (*Meijer et al. 1999*). High levels of immunoglobulin-A (IgA) and immunoglobulin (IgG) C. *pneumoniae* titres have been shown to correlated with mean annual AAA expansion and be significant independent predictors of AAA expansion (*Lindholt et al. 1999; Lindholt 2001*).

In an experimental animal model of AAA formation, macrophage influx into a rabbit abdominal aorta was insufficient to cause experimental aortic dilatation, however C. *pneumoniae* antigen influx induced both a macrophage influx and AAA formation, raising the possibility that C.*pneumoniae* may exert an effect on AAA formation through a macrophage mediated pathway (*Tambiah et al. 2001*).

Roxithromycin, a macrolide antibiotic with anti-Chlamydia effects, has been shown to decrease the clinical expansion rate of AAAs. The conclusion from this particular study was that C.*pneumoniae* induced and maintained a persistent inflammatory response that lead to AAA expansion (*Vammen et al. 2001*). Unfortunately whilst this study shows that Roxithromycin decreases AAA formation, the mechanism of its action was uncertain. The apparent slowing of AAA expansion could be due to a true antibiotic effect of Roxithromycin or the consequence of another property of the drug such as an anti-inflammatory activity. Indeed the tetracycline group of antibiotics have MMP inhibitory properties independent of their antimicrobial activity, and in one study have been shown to similarly decrease AAA expansion without having a significant effect on C.*pneumoniae* titres (*Petrinec et al. 1996b; Boyle et al. 1998; Mosorin et al. 2001*).

2.3.9 Autoimmunity

The fact that the inflammatory response associated with AAAs includes B and T lymphocytes (*Koch et al. 1990*), large amounts of immunoglobulin (*Brophy et al. 1991; Pasquinelli et al. 1993; Gregory et al. 1996*) and activated complement (*Capella et al. 1996*) suggests an autoimmune component to AAAs. It has been postulated that the formation of elastin-derived peptides may be an initiating factor in AAA formation (*Cohen et al. 1991; Cohen et al. 1992*). Indeed there are histological similarities with temporal arteritis where such elastin-derived peptides are also thought to be involved (*Cid et al. 1989*). Baydanoff demonstrated that anti-elastin antibodies are raised in AAA

patients (*Baydanoff et al. 1987*) leading Wills to postulate that elastin could potentially degenerate or have its structure altered by the ageing process forming elastin-derived peptides that could initiate AAA formation via the inflammatory cascade (*Wills et al. 1996*).

Tilson and Xia identified a matrix protein which is immunoreactive with Ig G isolated from AAA wall (*Tilson 1995; Xia et al. 1996*). Initial characterization of this putative autoantigen demonstrated homology to a microfibrillar-associated glycoprotein, which is an important component of the microfibrils that provide a structural scaffold for tropoelastin deposition during elastogenesis. Shah postulated that exposure of this putative autoantigen during elastolysis may incite an immune response and inflammatory cascade which ultimately leads to AAA formation (*Shah 1997*).

Despite the fact that several studies have demonstrated an association between the Class II major histocompatibility loci, D2, DR B1 and DR 2(15) and AAA formation (*Pasquinelli et al. 1993; Tilson et al. 1996; Rasmussen et al. 1997*), Walton and Yen, showed that in the vast majority of AAAs the B cell infiltrate and T cell receptor V beta repertoire were polyclonal and did not represent a restricted autoimmune response to a limited repertoire of tissue antigens, suggesting that an autoimmune basis for AAA formation was unlikely (*Walton et al. 1997; Yen et al. 1997*) .

2.3.10 Atherosclerosis

The fact that atherosclerosis results in AAA formation in only a small percentage of patients suggest that other factors may be involved in AAA formation. Despite sharing common risk factors such as smoking and hypertension and the fact that the two diseases may co-exist within the same individual, epidemiological data suggest that patients with aneurysmal disease are substantially older, overwhelmingly male, in better general health, experience lower post-operative mortality and postoperatively have a life expectancy similar to a normal population of the same age (*Tilson 1992; Shteinberg et al. 2000*). Additionally the incidence of diabetes and the lipid profile of AAA patients are normal whereas they are raised in AOD compared to the general population (*Norrgard et al. 1985; Louwrens et al. 1993*).

In addition, several histological and biochemical differences exist between aneurysmal and atherosclerotic aortic disease (*MacSweeney et al. 1994; Patel et al. 1995; Shteinberg et al. 2000*). In AAAs, the tunica media is thinner and its integrity destroyed, whilst in AOD tissue it is preserved to some extent. The elastic lamellae within the media and inner adventitia are destroyed in AAA disease and the VSMCs between the lamellae are deficient or reduced in AAA tissue compared to AOD derived tissue.

Three possible explanations exist to account for the strong association between atherosclerosis and AAAs.

1. Despite sharing common risk factors, AAA formation is unrelated to atherosclerosis and occlusive disease and is simply a unique clinical entity, with both developing and existing independently in the same individual. Tilson proposed that the common risk factors in both atherosclerotic occlusive disease and aneurysmal disease may mediate the promotion of either disease through unique disease specific mechanisms, depending on the constitutional susceptibilities of the individual (*Tilson 1992*).
2. A second explanation would be that atherosclerosis is a permissive factor required for AAA development. It is argued that AAAs may occur as a result of atherosclerotic plaque regression leaving a weakened aortic wall (*Zarins et al. 1990; Zarins et al. 1992; Zarins et al. 2001*). The infra-renal aorta is known to be relatively deficient in vasa vasorum and subsequently the tunica media is dependent on diffusion from the aortic lumen (*Wolinsky et al. 1967*). This paucity in vasa vasorum has been suggested as a factor that may explain the particular propensity of the infrarenal segment of the human aorta to develop early and severe atherosclerosis (*Reed et al. 1992*). It is argued that atherosclerotic plaques and superimposed thrombus form a mechanical barrier to the diffusion of substances into the media, thus weakening the media via nutritional deficiencies (*Wills et al. 1996*).
3. It has been suggested that atherosclerosis in the aneurysmal segment is a response to aortic dilatation rather than its cause, as atheromatous plaques preferentially form in regions of turbulence and low sheer stress(*Reilly et al. 1989; Cohen et al. 1990; Tilson 1992*).

The controversy surrounding the association between AAAs and atherosclerosis remains to be clarified, in particular to identify precisely if one is a prerequisite for the other, or whether indeed they are two distinct clinical entities just sharing common risk factors and a predilection for the infra renal aorta.

2.3.11 Summary of potential pathogenic mechanisms

In summary, AAA formation depends on the interplay between factors that either weaken the wall or increase the load on it together with the time scale over which they operate (*MacSweeney et al. 1994*). Advances in immunohistochemistry, biochemistry and molecular biology have demonstrated a complex multifactorial biophysical process that leads to an inflammatory response which in turn leads to increased media destruction and aortic remodelling. See **Fig 2.6 and 2.7** for schematic representation of pathogenesis of abdominal aortic aneurysms.

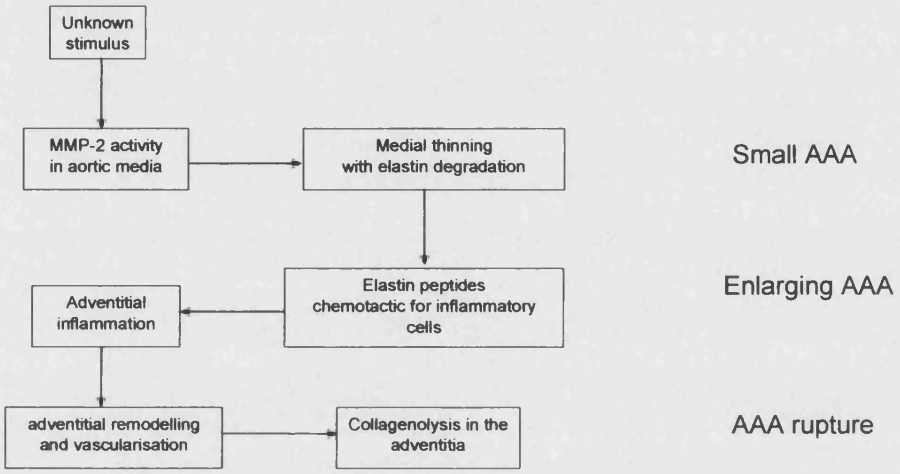

Figure 2-6 Pathogenesis of abdominal aortic aneurysms (*Powell 1998*)

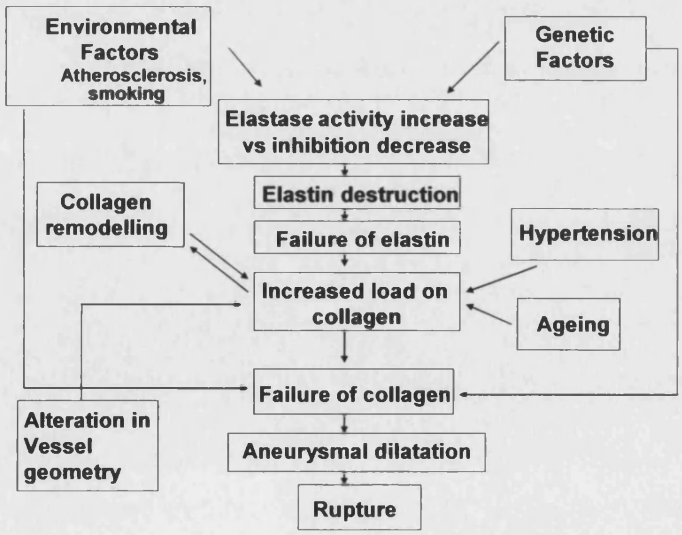

Figure 2-7 Alternative representation of pathogenesis of AAAs (*MacSweeney et al. 1994*)

2.4 Formulation of hypothesis

2.4.1 Arterial wall hypoxia

Arterial wall oxygenation has been the subject of several investigations. In 1944, Heuper suggested that artery wall hypoxia may have a role in the pathogenesis of atherosclerosis, and since then arterial-wall hypoxia has been a topic of investigation in conditions such as atherosclerosis, intimal hyperplasia and myointimal hyperplasia (*Hueper 1944; Heughan et al. 1973; Zemplenyi et al. 1989; Crawford et al. 1991; Martin et al. 1991; Bjornheden et al. 1999; Lee et al. 2000; Santilli et al. 2000*).

Wolinsky and Glagov demonstrated that arteries with the diameter and wall thickness of the rabbit aorta do not have a medial vasa vasorum (*Wolinsky et al. 1967*). Niinikoski demonstrated that a transarterial wall oxygen gradient is present in normal rabbit aortas with oxygen tensions falling from the adventitia and reaching a nadir at the junction of the inner one third and outer two thirds of the vessel wall. Oxygen tensions then slowly rise towards the lumen side (*Niinikoski et al. 1973*). This lead to the postulation that the outer two thirds of the artery wall is supplied with oxygen by the vasa vasorum and the inner one third of the artery wall is supplied by luminal diffusion of oxygen.

In atherosclerosis, both diffusion distances and the oxygen consumption of the arterial wall increase (*Vorp et al. 1996; Vorp et al. 1998*). Heughan demonstrated that the presence of an established atherosclerotic lesion eliminated the normal rise in oxygen tension found through the inner one-third of the vessel wall. Oxygen tensions progressively fell from the adventitia until the lumen was entered. In the presence of an atherosclerotic lesion, the luminal diffusion of oxygen to the inner one-third of the artery wall was impaired (*Heughan et al. 1973*). Despite the attractiveness of this theory the following question remains: What happens before atherosclerotic lesion formation to initiate and maintain the formation of atherosclerotic lesions?

Martin postulated that thrombosis of the vasa-vasorum leading to arterial wall hypoxia, is an initial lesion in atherosclerosis (*Martin et al. 1991*). Additional evidence from animal studies has shown that destruction of vaso-vasorum alters the trans-mural oxygen tension and leads to vessel wall hypoxia (*Santilli et al. 2000*). Crawford reported that hypertension causes artery wall hypoxia and could lead to atherosclerosis through the generation of oxyradicals (*Crawford et al. 1991*). Santilli has shown that risk factors for atherosclerosis including diabetes, hypertension, cigarette smoking, ageing, and artery bifurcation lead to artery-wall hypoxia before the formation of an

atherosclerotic lesion (*Santilli et al. 1992, 1993; Santilli et al. 1995; Santilli et al. 1998*).

The oxygen concentration in the arterial wall has been measured directly in animals and *in vitro* and *in situ* systems and these measurements demonstrate that partial pressure of oxygen (pO_2) decreases with the distance from the lumen and adventitia, reaching a minimum of 20 to 50 mm Hg in the media (*Niinikoski et al. 1973; Crawford et al. 1988; Zemplenyi et al. 1989*). These measurements have all demonstrated that a trans-arterial wall oxygen gradient is a normal physiological finding, which is exaggerated in an atherosclerotic vessel.

Data from human studies and in particular *in vivo* measurements are limited. Bjornheden described a hypoxia marker, 7- (4'-(2-nitroimidazol-1-yl)-butyl) theophylline (NITP) with potential to assess hypoxia *in vivo*. The system is based on a hypoxia marker originally developed by Hodgkiss to detect hypoxia in tumours (*Hodgkiss et al. 1991*). In this system a nitroimidazole derivative with a theophylline ligand is taken up intracellularly and undergoes nitroreduction and oxidation (mainly by cytochrome P_{450} reductase) in a futile cycle provided oxygen is present. In hypoxic conditions, the cycle is broken and aggressive radicals form which bind to cellular constituents and become trapped. The theophylline residues may then be detected by immunological methods. The main advantage with this method is that NITP may be administered *in vivo*, which makes it possible to assess hypoxia in arterial tissue in the intact animal.

In animal (rabbit) *in vivo* experiments, experimental atherosclerosis was induced in rabbits by a combination of cholesterol-enriched diet and mechanical injury and NITP solution was administered intraperitoneally. In the rabbit aorta, distinct NITP-associated immunofluorescence was observed in atherosclerotic lesions that were >400 to 500 μm thick. The fluorescence was localized intracellularly to foam cells that occupied a 200- to 300-μm-wide zone at a distance of 200 to 300 μm from the luminal surface and from the adventitial vasa vasorum. In *in vitro* conditions, both foam cells and smooth muscle cells accumulated NITP. Smooth muscle cells bound NITP at a pO_2 < 2 to 3 mm Hg, whereas foam cells bound the hypoxia marker at a somewhat higher pO2 (*Bjornheden et al. 1999*). The interpretation of these results was that the arterial tissue in the zones of NITP immunofluorescence was hypoxic and the presence of the bound marker corresponded to a pO_2 of 30 to 40 KPa (2 to 3 mm Hg) in the periphery of these zones. To my knowledge this is the only demonstration that hypoxic zones do occur within atherosclerotic plaques in rabbits when the lesions exceed certain

dimensions and at a depth that is readily reached in humans. It seems likely that the hypoxia in these areas reflect an impaired diffusion capacity due to the thickness of the plaque, together with an increased demand in the metabolically active foam cells.

The fluid mechanics within AAA wall are conducive to a build up of laminated intraluminal thrombus (ILT) (*Adolph et al. 1997; Vorp et al. 1998*). The role of ILT in AAA pathogenesis and progression is unknown although previous studies have suggested a relationship between the presence of ILT and AAA size, enlargement and rupture (*Inzoli et al. 1993; Wolf et al. 1994; Satta et al. 1996; Vorp et al. 1996*). Previous interest has focussed on the possibility that ILT forms a mechanical protective layer although recent interest has looked at the possibility that ILT serves as a mechanical barrier to oxygen diffusion and leads to a reduction in aortic wall strength and stiffness (*Inzoli et al. 1993; Vorp et al. 1996*).

Vorp hypothesized that ILT serves as a barrier to normal physiological oxygen diffusion from the lumen to the inner layers of the aortic wall rendering the intima and inner media anoxic (*Vorp et al. 1996; Vorp et al. 1998*). Assessment of this hypothesis using an order-of-magnitude analysis of the oxygen diffusion through the ILT layer compared to the oxygen consumption rate required for normal aorta revealed an oxygen flow rate 20 times less than that required for normal VSMC function in AAA. This analysis suggested that ILT may offer a significant barrier to O_2 diffusion through the AAA wall, leading to anoxia, necrosis and diminished resistance by the aortic wall to physiologic distending pressure (*Vorp et al. 1996*). In further computational analysis of oxygen flow to the AAA wall, the effects of the ILT thickness and AAA bulge diameter were assessed. In this study ILT thickness was critical in determining the amount of attenuation of oxygen flow to the AAA wall, while bulge diameter had little effect (*Vorp et al. 1998*).

Preliminary data collected by Vorp support the possibility that ILT-induces AAA wall hypoxia. In brief, eight patients with AAA had their pO_2 measured intraoperatively with a custom needle type polarographic pO_2 electrode. The pO_2 measurements were normalised by the intraluminal pO_2 to account for patient variability. The results indicated that the normalised pO_2 within the AAA wall in patients with ILT was significantly less than that within the AAA wall in patients without ILT (*Vorp et al. 1998; Vorp et al. 2001*).

2.4.1.1 Effects of hypoxia

2.4.1.1.1 Vascular tissue

Hypoxia is recognised as a specific stimulus for gene expression, and has been shown to modulate the expression of transcription factors such as Hypoxia inducible factor -1 (HIF-1), growth factors and angiogenic factors such as vascular endothelial growth factor (VEGF), platelet derived endothelial growth factor (PDEGF), endothelin (ET-1), and nitric oxide synthase (NOS) (*Sakuda et al. 1992; Kourembanas et al. 1998; Berse et al. 1999; Chiarugi et al. 1999; Dachs et al. 2000*).

Hypoxia has been shown to decrease elastin and collagen synthesis in cultured aortic endothelial cells, VSMCs and fibroblasts (*Stavenow et al. 1987; Durmowicz et al. 1991; Herrick et al. 1996; Steinbrech et al. 1999*). Furthermore, the collagen that is synthesised by hypoxic cells is abnormal because oxygen is needed for the hydroxylation of praline (*Herrick et al. 1996*). Therefore the extra-cellular matrix synthesis by the hypoxic vascular wall is likely to be impaired or results in abnormal matrix which could lead to wall weakening (*Vorp et al. 2001*).

2.4.1.1.2 Hypoxia and MMPs

Macrophages exposed to hypoxia show an increase in elastase production (*Campbell et al. 1983*). MMP-9 has been shown to contain consensus hypoxia sensitive AP-1 and NFκB sites in its promoter region (*Himelstein et al. 1998*). In mice exposed to hypoxia, MMP-2 and MMP-9 protein levels increased by 2 days (*Zaidi et al. 2002*). Similarly, in pulmonary interstitial tissue harvested from hypoxic exposed rabbits rabbit and human keratinocytes exposed to 2% oxygen, MMP-9 expression and protein levels increased (*O'Toole et al. 1997; Miserocchi et al. 2001*). However in one study using mouse fibroblasts, MMP-9 expression and activity decreased after exposure to hypoxia (*Saed et al. 2000*).

Other studies have demonstrated an increase in expression of MMP-2 in rat hepatocytes subjected to hypoxia (*Chen et al. 2000*). In one study using human trophoblastic and breast cancer cells, hypoxia had no effect on either MMP-2 or MMP-9 expression, but their activity was increased due to a decrease in TIMP-1(*Canning et al. 2001*). These findings suggest reduced oxygen conditions may shift the balance between MMPs and their inhibitors favouring increased MMP activity.

2.4.2 Xanthine oxidoreductase (XOR)

2.4.2.1 Background information

Xanthine oxidoreductase is a homodimer of 150kDa subunits existing as two interconvertible forms, xanthine dehydrogenase (XDH; EC 1.1.1.204) and xanthine oxidase (XO; EC 1.1.3.22). The two enzyme forms and their reactions are often referred to as xanthine oxidoreductase (XOR) activity (*Pritsos 2000*).

XOR is a cytosolic and membrane-bound complex molybdoflavoprotein with each 150kDa subunit containing the following active redox centres (*Bray et al. 1996*).

1. 1 molybdenum centre with a pterin co-factor
2. 2 iron sulphur centres (Fe/S I and Fe/s II) and,
3. 1 Flavin adenine dinucleotide (FAD) centre.

The gene for xanthine oxidase is located on chromosome 2 and codes for a sequence of 1330 amino acids (*Xu et al. 1994*). The enzyme is synthesized as xanthine dehydrogenase (XDH), which accounts for 90% of the total activity in healthy tissue (*McCord 1985*). The functional distinction is the preference of each for differing terminal electron acceptors. XDH is characterized by high xanthine/NAD^+ activity and low xanthine/O_2 activity, whereas XO is characterized by high xanthine/ O_2 activity and low xanthine/ NAD^+ activity (*Nishino et al. 1997; Pritsos 2000*). This difference is due to the absence of a NAD binding site as a result of destabilization of the FAD redox site in the oxidase form. However, the precise structural difference between the two forms of the enzyme remains elusive (*Nishino et al. 1997*). Conversion of xanthine dehydrogenase into xanthine occurs by two mechanisms:

1. Thiol oxidation of the protein molecule (reversible), or
2. Proteolysis (irreversible).

This conversion readily takes place during isolation procedures unless thiol reducing agents and protease inhibitors are included in isolation buffers (*Nishino et al. 1997; Pritsos 2000*).

2.4.2.2 Tissue and cellular distribution of XOR

The expression of XOR is tissue and cell specific. Small amounts of XOR are present in various organs, with the highest levels of catalytically active enzyme being observed in the liver, intestine, lung and heart (*Terao et al. 1997; Pritsos 2000*). Substantial differences in organ distribution of XOR activity exist between mammalian species making it difficult to extrapolate animal study results to human physiological and pathological events (*Pritsos 2000*). Human liver and intestine have the highest XOR

activity of any tissues and this is largely due to the XOR-rich parenchyma cells of these tissues (*Pritsos 2000*).

XOR has also been shown to be present in microvascular endothelial cells which may account for the extremely low tissue activity seen when relatively large pieces of tissue are homogenized for enzymatic activity or blotting (*Jarasch et al. 1986; Moriwaki et al. 1993; Vickers et al. 1998*). In addition XOR has been shown to be released into the systemic circulation from the liver and intestine during reperfusion injury (*Yokoyama et al. 1990; Tan et al. 1995*). XOR has also been localized to the endothelial cell plasma membranes, binding via interaction with surface glycosaminoglycans (*Adachi et al. 1993*).

2.4.2.3 XOR and vascular tissue

Levels of both XOR and anti-XOR antibodies are known to be elevated in plasma of atherosclerotic patients and the presence of the enzyme has been demonstrated in vessel walls and atherosclerotic plaques (*Harrison et al. 1990; Swain et al. 1995; Mohacsi et al. 1996; Patetsios et al. 1996; Patetsios et al. 2001*).

Swain investigating the presence of redox active iron in human atherosclerotic material, demonstrated that ferroxidase activity attributable to XOR was present in both carotid endarterectomy plaques and aneurysm tissue (*Swain et al. 1995*). Patetsios' investigation of uric acid (the end product of XOR activity) and its role in " response to injury theory of atherosclerosis" demonstrated that both AAA tissue and carotid endarterectomy plaques contained significantly more uric acid than non-atherosclerotic arteries obtained from young cadaveric organ donors. Immunohistochemistry localised XOR to the intima and inner media of the artery wall. The conclusion from this study was that increased XOR activity within an artery wall may lead to the deposition of uric acid and trigger arterial injury (*Patetsios et al. 1996*).

In a subsequent study using carotid endarterectomy plaques and control carotid arteries from cadaveric organ harvests, significantly higher concentrations of uric acid were seen in atherosclerotic plaques. Control carotid arteries stained positive for XOR, but staining was less than that of carotid plaque. The staining was localized to both the intima and media, being more positive in the media whereas carotid plaque demonstrated greater intimal staining. The carotid plaques were irregularly stained, with endothelial cells and smooth muscle cells staining positive for XOR in the intima. Uric acid and XOR co-localised within the specimens. The conclusion from this study was that injury to the endothelial and smooth muscle cells initiated the conversion of XDH to XO. Increased XO activity results in the production of uric acid from purine

precursors with the resultant generation of free radicals that may injure the artery (*Patetsios et al. 2001*).

2.4.2.4 Reactions of XOR

Xanthine oxidoreductase has long been associated with the mechanism of gaining and losing electrons in an apparent electron shuttling capacity. The enzyme has a wide specificity for reducing substrates, although its' physiologically role is in the conversion of hypoxanthine to xanthine and xanthine to uric acid. Both forms of the enzyme can reduce molecular oxygen, although XDH can reduce NAD+ which is its preferred electron acceptor (*Pritsos 2000*).

Reduction of oxygen leads to superoxide and hydrogen peroxide and it is this potential to generate reactive oxygen species that lead to interest in this enzyme in the pathogenesis of ischaemia re-perfusion injury (*McCord 1985*). Recent interest has focussed on the ability of XOR to reduce inorganic nitrates and nitrites and produce nitric oxide (NO) (*Millar et al. 1998*). The simultaneous production of superoxide and NO by XOR leads to the formation of peroxynitrite, a potent oxidising, nitrating and hydroxylating agent (*Godber et al. 2000b*).

It is to the Molybdenum centre that most reducing substrates donate their electrons (*Pritsos 2000*). The only exception to this is when NADH is the reducing substrate, in which case the electrons are transferred at the FAD site (*Pritsos 2000*). The electrons are ultimately transferred to either molecular oxygen (xanthine oxidase) or NAD (xanthine dehydrogenase), which serve as terminal electron acceptors.

2.4.2.4.1 Purine metabolism

XOR is involved in the oxidation of hypoxanthine to xanthine and the subsequent oxidation of xanthine to uric acid. XDH reduces NAD by a direct two-electron reduction while XOR reduces molecular oxygen by a single electron. These reactions are inhibited by blocking the molybdenum centre of the enzyme with purine analogues such as allopurinol and oxypurinol (*Pritsos 2000*).

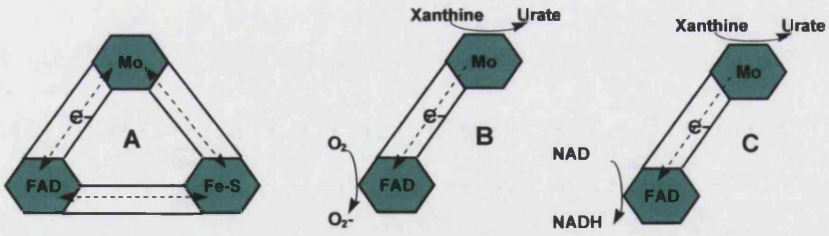

Figure 2-8 Electron shuttle of XOR

(A) **demonstrates a generalised scheme of electron transport utilising the three redox centres of the enzyme Mo molybdopterin, flavin centre (FAD) and the iron sulphur centres (Fe-S).** (B) **Details the xanthine to urate reaction by XO using molecular oxygen as the electron acceptor and** (C) **details of xanthine to urate reaction by XDH using NAD as the electron acceptor.**

2.4.2.4.2 NADH

Human XOR purified from breast milk is characterized with a remarkably low activity towards hypoxanthine, xanthine and other conventional reducing substrates which donate their electrons directly to the molybdenum site (*Sarnesto et al. 1996; Harrison 1997*). NADH is an alternative substrate that donates electrons directly at the FAD site. Both forms of the enzyme are able to utilize NADH as a substrate, but it is particularly XDH which is able to perform this reaction (*Sanders et al. 1997*).The oxidation of NADH to NAD+ is shown in **Figure 2.9** with the electron again being given to oxygen to produce the superoxide radical.This reaction is not affected by the molybdenum site inhibitors allopurinol and oxypurinol in the presence of oxygen but is inhibited by NAD+ and also by the FAD specific inhibitor diphenyliodonium (DPI) (*Sanders et al. 1997*).

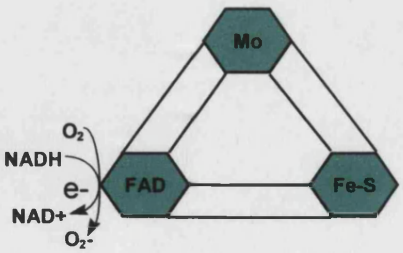

Figure 2-9 Oxidation of NADH to NAD+ at the Flavin centre and the generation of superoxide radical

2.4.2.4.3 Nitrates/nitrites

Recent interest has focused on the ability of XOR to generate nitric oxide (NO) from nitrates (NO_3^-) and nitrites (NO_2^-) (*Zhang et al. 1997; Millar et al. 1998; Zhang et al. 1998; Godber et al. 2000b*). In an oxygen rich environment, XOR favours its ability to oxidize NADH or xanthine generating superoxide radicals. In oxygen depleted tissue, NADH or xanthine can donate electrons which rather than forming superoxide, reduce NO_3^- to NO_2^-. The terminal electron transfer occurs at the molybdenum centre when oxygen is restricted. Activity is completely blocked by oxypurinol at the molybdenum site. The donated electron may be competed for by the NO_3^- at the molybdenum centre and residual oxygen in the incubation medium at the FAD centre. Activity in favour of NO_2^- production can be enhanced by blocking the leak of electrons to oxygen using the FAD-site inhibitor diphenyleneiodonium (DPI) (*Blake et al. 1997; Millar et al. 1997*). Millar demonstrated that XOR has NO_2^- reducing activity when exposed to an oxygen limited environment. Nitrite reductase activity can utilise either xanthine at the molybdenum site or NADH at the FAD site as electron donors and the electron is then passed to NO_2^- at the FeS centre generating nitric oxide (NO)(*Millar et al. 1998*). The generation of both nitric oxide and superoxide radicals by XOR can lead to their interaction and generation of the potent oxidant, peroxynitrite, which is believed to play a pivotal role in the pathogenesis of oxidant stress related conditions such as atherosclerosis (*Godber et al. 2000b*).

2.4.2.5 Hypoxia and XOR

The relationship between XOR and hypoxia involves both transcriptional and post-translation modulation. Hypoxia has been shown to increase the expression of XOR at a transcription level in several studies (*Hassoun et al. 1994; Lanzillo et al. 1996; Terada et al. 1997; Hassoun et al. 1998*). The mechanism by which oxygen tension effects XOR expression appears to be complex, however the 5' flanking region of the XOR gene has been shown to contain an AP-1 motif, 3 potential AP-2 sites and a putative hypoxia-inducible factor-1-binding site (*Hassoun et al. 1998*).

In addition, studies also show that hypoxia increases XOR activity without a concomitant de novo increase in XOR synthesis or mRNA levels, suggesting a post-translational effect of hypoxia (*Terada et al. 1992; Cote et al. 1996; Poss et al. 1996; Terada et al. 1997; Hassoun et al. 1998*).

2.4.3 Oxidative stress

The term oxidative stress refers to a situation in which cells are exposed to excessive levels of either molecular oxygen or chemical derivatives of oxygen such as reactive oxygen species (ROS) (*Zalba et al. 2000*).

A free radical is any species capable of independent existence that contains one or more unpaired electrons. Examples are the oxygen-cantered radicals superoxide and hydroxyl, the glutathione radical and nitric oxide, a radical in which the unpaired electron is delocalised between both atoms (*Halliwell 1993*).

A normal chemical bond consists of a pair of electrons, opposite in spin and sharing a single molecular orbital. A free radical is a molecule containing an odd number of electrons and thus may be considered to contain an open bond, rendering it chemically reactive. Radicals can react with other molecules in a number of ways. Firstly, when two radicals meet they can combine their unpaired electrons to form a covalent bond. Secondly, they can react with non-radicals by either donating the unpaired electron (reduction) or they may take an electron from another molecule (oxidation). Thirdly, a radical may attach itself to a non-radical. In general, when any of these reactions take place the non-radical species becomes a radical. This usually has a knock on effect creating a chain reaction of these events. Superoxide, hydroxy radical (OH -) and nitric oxide are examples of free radicals whilst others, such as hydrogen peroxide (H_2O_2) and peroxynitrite (OONO-), are not radicals but are biologically reactive species (*Zalba et al. 2000*).

In normal individuals, the generation of ROS appears to be in balance with anti-oxidant defences, although there may be a slow cumulative oxidative damage that contributes to the ageing process. Humans appear not to have a great reserve of anti-oxidant defences, perhaps because ROS play some useful metabolic role. An imbalance between ROS and anti oxidant defences in favour of formation of ROS leads to oxidant stress. This can happen either if anti-oxidant levels are depleted and/or if ROS formation is increased. Most cells can tolerate mild oxidative stress, which often leads to increased synthesis of anti-oxidant defence systems. However, severe oxidative stress produces derangements of cell metabolism, including DNA strand breakage, increased intra-cellular Ca^2+, damage to membrane ion transporters, peroxidation of lipids, depletion of NAD+ and ATP and the activation of proteases. Oxidant stress has been implicated in a vast number of diseases including atherosclerosis, haemorrhagic shock, ischaemia-reperfusion injury, cancer, rheumatoid arthritis and inflammatory bowel disease (*Halliwell 1993; Zalba et al. 2000*).

Metabolism of ROS is tightly controlled. Dismutation of superoxide by superoxide dismutase (SOD) produces the more stable hydrogen peroxide, which in turn is converted to water by catalase and glutathione peroxidase. Removal of excess superoxide by SOD enzymes is an important physiological antioxidant defence mechanism. It appears that one of the most important roles of SOD is the prevention of the reaction of superoxide with nitric oxide to form peroxynitrite (*Wolin 2000*). Superoxide reacts with nitric oxide at a rate which is 3 times the rate of reaction with SOD (*Goldstein et al. 1995*). When the level of NO increases and approaches the local concentrations of SOD, nitric oxide is able to compete with SOD for the scavenging of superoxide to form significant amounts of peroxynitrite (*Wolin 2000*).
The tight regulation of both production and removal of ROS makes fluctuations in their levels transient (*Griendling et al. 2000*). On the whole, however, superoxide and hydrogen peroxide have limited chemical reactivity and interest has focused on their ability to generate more reactive species such as peroxynitrite (*Zalba et al. 2000*). Peroxynitrite is much more reactive than NO or superoxide and will cause diverse chemical reactions in biological system including nitration of the tyrosine residues of proteins, triggering of lipid peroxidation, inhibition of mitochondrial electron transport, and oxidation of biological thiol compounds (*Maeda et al. 1998a*).

2.4.3.1 Vascular oxidant stress

In the vascular wall, increases in oxidant stress are thought to alter several important physiological functions, including regulation of blood flow, platelet aggregation, leukocyte adhesion and cellular growth. These phenomena ultimately modulate vessel diameter, remodelling and lesion formation (*Zalba et al. 2000*).
Three enzyme systems produce reactive oxygen and nitrogen species in the vascular wall: NADH/NADPH oxidase, xanthine oxidoreductase, and endothelial nitric oxide synthase (NOS) (*Griendling et al. 2000; Zalba et al. 2000*)

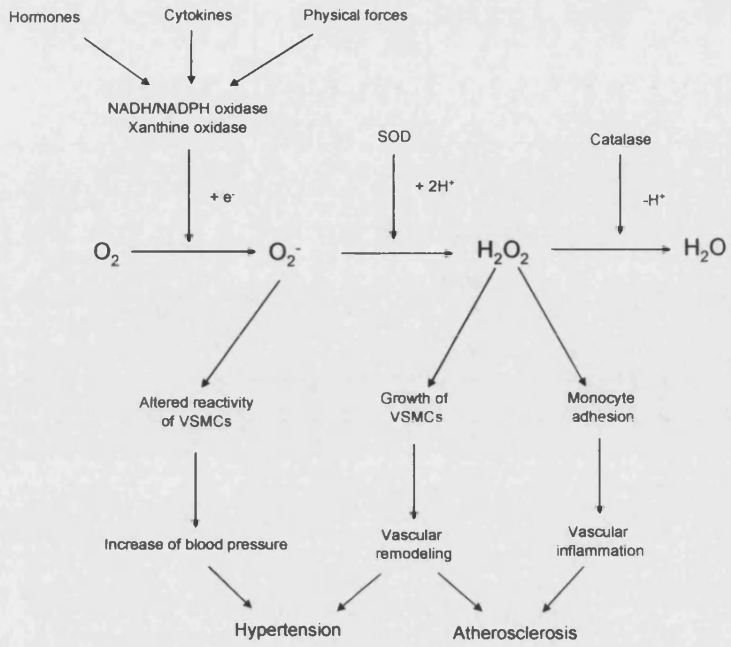

Figure 2-10 Representation of potential roles of oxidant stress in vascular disease

2.4.3.2 Oxidant stress and possible role in aneurysms

2.4.3.2.1 Evidence of oxidant stress in aortic aneurysms

The possibility that oxidant stress may contribute to the pathogenesis of AAA formation is supported by studies that have shown the AAA tissue has reduced levels of ascorbic acid and antioxidant enzyme activities compared to non-aneurysmal tissue (*Dubick et al. 1999*). Furthermore, plasma levels of vitamin E, another anti-oxidant were found to be markedly reduced in patients with AAA but not in patients with coronary artery disease in the absence of AAA (*Sakalihasan et al. 1996a*). These intriguing observations would support the hypothesis that oxidant stress is potentially increased in AAA tissue.

Since the conduction of our study, one published report has appeared in the literature about oxidant stress in AAAs. In this study, segments of AAA tissue were compared to adjacent non-aneurysmal tissue within the same patient. The study found that superoxide levels (measured by lucigenin-enhanced chemiluminescence) were higher in the AAA segments compared with the adjacent non-aneurysmal aortic segments, and the source of the superoxide was localised to infiltrating inflammatory cells and to VSMCs. Formation of thiobarbituric acid-reactive substances and conjugated dienes, two indices of lipid peroxidation, were also increased in AAA compared with non-aneurysmal segments. Finally, immunostaining for nitrotyrosine was significantly

greater in AAA tissue, all suggesting that oxidant stress was associated with AAA formation (*Miller et al. 2002*).

2.4.3.2.2 Potential mechanisms of oxidant stress in AAA formation

The potential mechanisms by which oxidant stress may lead to AAA formation include;

1. Apoptosis. Reactive oxygen species have been reported to induce apoptosis of VSMCs which is a well described feature of AAA tissue (*Holmes et al. 1996; Lopez-Candales et al. 1997; Henderson et al. 1999*).
2. Increased gene expression. Free radicals and ROS are known to influence the expression of a number of genes and signal transduction pathways, as well as acting as sub-cellular messengers for certain growth factors. The Mitogen-Activated Protein kinases (MAP kinase) and the Nuclear factor kappa-B (NFκB) signal transduction pathway and the transcription factors AP-1 are all oxidant sensitive (*Allen et al. 2000; Griendling et al. 2000*). MMP-1, MMP-3 and MMP-9 genes have AP-1 and NFκB binding elements in their promoter regions, and NFκB binding sites are also present in aneurysm associated cytokine genes including TNFα, IL-1, IL-6 which thus confer oxidant sensitivity to these genes (*Huhtala et al. 1991; Sato et al. 1993; Vincenti et al. 1998; Allen et al. 2000; Bond et al. 2001*).The presence of an NFκB site in the MMP-9 promoter and an NFκB-like element in the MMP-1 prompted Bond to investigate the effects of IL-1α (potent activator of NFκB) on the secretion of MMP-1 and MMP-9. This study showed that IL-1α potently activated NFκB and upregulated the expression of MMP-1 and MMP-9. Over expression of IκB (an inhibitor of NFκB) almost completely inhibited the expression of these MMPs (*Bond et al. 2001*).Changes in the cellular redox state have been demonstrated to modulate the expression of MMP-1 in human dermal fibroblast cultures (*Brenneisen et al. 1997*), MMP-2 in cultured bovine aortic endothelial cells (*Inoue et al. 2001*) and MMP-9 in bovine aortic endothelial cells (*Uemura et al. 2001*). Galis showed that N-acetyl-L-cysteine, an anti-oxidant, decreased MMP-9 expression in hypercholesterolaemic rabbits (*Galis et al. 1998*).
3. Activation of MMPs. MMPs are secreted in a latent zymogen form in which the prodomain shields the catalytic site. This conformation of zymogens is maintained due to thiol interactions between cysteine residues in the prodomain and the zinc atom present in the catalytic site of all MMPs (*Ye et al. 1998*) .The critical step in the activation process, described as opening of the 'cysteine switch', is followed by a series of autocatalytic cleavages resulting in the complete excision of the pro-peptide and activation of the enzyme. Reactive oxygen species are known to react with thiol

groups such as those involved in preserving MMP latency, so they could modulate the activity of MMPs (*Rajagopalan et al. 1996*). Rajagopalan demonstrated that pro-MMP-2 and pro-MMP-9 secreted into the media of cultured human smooth muscle cells are activated by ROS (*Rajagopalan et al. 1996*). Similar work by Siwik, using rat cardiac fibroblast demonstrated identical results using both superoxide and hydrogen peroxiden (*Siwik et al. 2001*). Buhimschi using human foetal membranes demonstrated that superoxide significantly affected MMP-9 activity. Interestingly the glutathione precursor N-acetylcysteine (an anti-oxidant) dramatically inhibited MMP-9 activity (*Buhimschi et al. 2000*). Similar results were seen in bovine aortic cells (*Uemura et al. 2001*), and hypercholesterolaemic rabbits (*Galis et al. 1998*). Peroxynitrite has been shown to activate MMPs (*Rajagopalan et al. 1996; Okamoto et al. 1997; Maeda et al. 1998a*). Peroxynitrite seems to interact with a single cysteine residue in the propeptide autoinhibitory domain, or so-called cysteine switch of pro-MMPs, thus transforming pro-MMPs into their active conformation. However in one study peroxynitrite was shown to decrease the activity of MMP-2 (*Owens et al. 1997*).

4. Inactivation of MMP inhibitors. In addition to its effects on MMPs, peroxynitrite has been shown to inactivate TIMPs and alpha1-proteinase inhibitor, a major protease (neutrophil elastase) inhibitor in human plasma the net effect being accelerated tissue degradation (*Frears et al. 1996; Maeda et al. 1998a*)

2.4.4 Hypothesis

The hypothesis tested was that hypoxia leads to increased elastolysis and potential AAA formation either directly or via a XOR/oxidant stress mediated pathway.

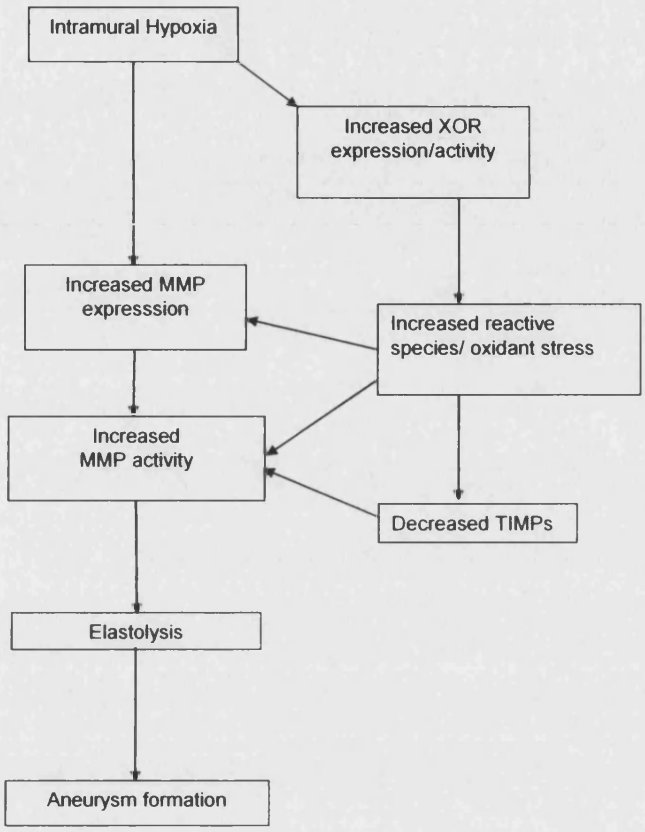

Figure 2-11 Diagrammatic representation of hypothesis

GENERAL METHODS

Chapter 3 Materials and Methods

3.1 Ethics and consent

The Medical Research Ethics Committee of The Royal United Hospital Bath, approved all tissue-harvesting procedures and subsequent laboratory studies. Informed consent for the use of surgical tissue specimens was obtained from each patient. Control aortic tissue was obtained from cadaveric organ donors (Southmeads Hospital, Bristol). Informed consent for tissue collection was obtained from relatives in all cases.

3.2 Tissue collection

Aortic wall biopsy specimens ($2cm^2$) were excised from the anterior aortic wall at the time of aortotomy during operative aneurysm repair or from the aortic cuff of cadaveric organ donors (*Crowther et al. 2000b*).

3.3 Histology and immunohistochemistry

3.3.1 Tissue preparation

Specimens were stored in formal saline, 40% volume formaldehyde in 0.9% NaCl for seven days. Samples were processed using a Shandon hypercenter II wax-embedding station. Each tissue sample was enclosed within a plastic cassette and subjected to increasing concentration of ethanol, 70%, 80%, 95% and then absolute ethanol (3x) with a residence time of 1 hour at each concentration. This was followed by three one-hour washes in xylene before embedding in molten wax at 60°C. Wax blocks were stored at room temperature. Sections (5μm) were cut using a microtome blade cutter (Leitz) and placed on a microscope slide and left to dry overnight at 50°C. The microscope slides were previously coated with poly-L-lysine to aid adhesion and to avoid loss of tissue sections during staining procedures. Sections were dewaxed in xylene and rehydrated using a graded series of alcohol solutions through to distilled water or 1x PBS (*Appendix*).

3.3.2 Haematoxylin and Eosin staining

Sections were immersed in water for 5 minutes and then placed in Harris' haematoxylin for 5 minutes. Sections were transferred to an acid alcohol solution (1%) for 1 minute, water for a further 5 minutes, eosin for 30 seconds and then water for 5 minutes. Sections were then placed in IMS (2 x 30 seconds) and 100% xylene (2 x 30 seconds) and then mounted in DPX (*Appendix*)

3.3.3 Immunohistochemistry

3.3.3.1 Localisation of XOR and in human aortic wall sections

Sections were processed for XOR detection by use of the Vectastain ABC-Alkaline Phosphatase Kit for detecting rabbit immunoglobulin G. Sections were washed (PBS, 5 minutes x 2) and incubated with 200µl of blocking buffer (5 mg bovine serum albumin (BSA), 333 µl normal goat serum, 10mls PBS) for 30 minutes. After a wash in PBS, 200µl of the primary antibody (Polyclonal rabbit anti-bovine XO, Chemicon) (1:50 – 1:200 in blocking solution) was added and sections incubated overnight at 4°C in a humidified atmosphere. The sections were washed (PBS, 5 minutes x 2) and incubated with biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Vectastatin® (200µl per slide)) for 1hr. Following washing, sections were incubated with ABC-Alkaline Phosphatase conjugate (AP) complex (1:100) (Vectastain, Vector Lab) for 30mins, and finally with Fast Red TR/Napthol AS-MX in distilled water (Sigma) at room temperature. The reaction was followed until a brown colour appeared and then terminated by the addition of copious amounts of Ultra pure water.
Sections were counterstained with Mayer's Haematoxylin for 1 minute and washed in water until the blue nuclear colouration was distinct. The sections were mounted in Aquamount (Merck)(*Appendix*).

3.3.3.2 Immunohistochemical detection of 3 nitrotyrosine (3-NT) in human aortic wall sections

Formation of free and protein 3-nitrotyrosine derivatives has been used as a probe for peroxynitrite formation. (*Ischiropoulos 1998; Tarpey et al. 2001; Miller et al. 2002*). An identical technique as used for the immunodetection for XOR was employed. Anti-nitrotyrosine (Upstate Biotechnology) was used at a dilution of 1:100. The sections were washed and incubated with biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Vectastatin® (200µl per slide)) for 1hr (*Appendix*).

3.4 Western blotting for determination of proteins

3.4.1 Sample preparation

Additional aortic samples were placed in Hank's balanced salt solution (HBSS). Approximately 100 mg (wet weight) of aortic tissue was added to 1.5 mls of Homogenisation buffer (50mM KPO4 pH 7.3, 1mM EDTA, 1mM PMSF, Pepstatin A (1 µgml-1), Antipain (1 µgml-1), Leupeptin (1 µgml-1), Aprotinin (1 µgml-1) and the mixture homogenised with a mechanical homogenizer. The homogenates were

centrifuged at 10,000 rpm, for 10mins at + 4°C and the supernatant retained and stored at -70 °C (*Beckman et al. 1989*)(*Appendix*).

3.4.2 Bradford assay for total protein estimation

Total protein was estimated using the Bio-Rad Protein Assay. This is a dye-binding assay based on the differential colour change of a dye (Coomassie Brilliant Blue G-250) in response to various concentrations of protein (*Bradford 1976*). The dye binds to primarily basic (especially arginine) and aromatic amino acid residues. Briefly, several dilutions of bovine serum albumin (protein standard II) containing 0.625 - 80μg/ml were prepared. 80μl of diluted standards were added to 20μl of dye reagent per well of a 96 well plate. All standards were performed in triplicate. After 5mins the optical density at 595nm was measured on a microplate reader. A standard plot was constructed using the corresponding OD values and the protein concentrations. The amount of protein in the unknown sample is determined by interpolation, reading the concentration of protein on the standard curve that corresponds to its absorbance (*Appendix*).

3.4.3 Polyacrylamide gel electrophoresis SDS-PAGE

To confirm XOR protein within aortic samples, an antibody raised against bovine buttermilk XOR was used to detect immobilised proteins by Western blot analysis. Typically, proteins are electrophoresed through a SDS polyacrylamide gel under reducing conditions before transfer to a nitrocellulose membrane. This membrane is then probed for the appearance of XOR protein using the anti-XOR antibody and a chemiluminescence detection technique. The appearance of a 150 KDa band is typical of XOR protein but some preparations show the typical degradation products of XOR (*Amaya et al. 1990; Harrison 1997; Nishino et al. 1997*).

Proteins in a solution of negatively charged detergent (SDS) are unfolded and negatively charged. The addition of a reducing agent such as mercaptoethanol or dithiothrietol breaks di-sulphide bonds. Applying a current across the gel causes the charged proteins to migrate toward the positive anode according to their molecular weight, with the larger proteins being retarded by the gel.

Acrylamide gels (8%) were prepared according to Laemmli (*Laemmli 1970*). An equal volume of 2x reducing sample buffer was added and the solution boiled at 100°C for 3 minutes. 10μl of rainbow markers (molecular weights, 250-10kD) (Amersham) were mixed with an equal volume of 2x reducing loading buffer. The samples were centrifuged at 10,000 rpm (Biofuge, Heraeus Instruments) for 5mins. The gel plates were placed in the ATTO electrophoresis set up and filled with running buffer

containing 25mM Tris base, 190mM glycine and 0.1% SDS (w/v) pH 8.3. An equal volume (10-30 μL) of each sample containing equal amounts of total protein (10-30μg) was carefully loaded into the gel wells. The gel was then subjected to electrophoresis at a constant voltage of 100V for approximately 2 hours, or until the dye reached the base of the gel (*Appendix*).

3.4.4 Western blotting

Following electrophoresis, the gels were removed and incubated in blotting buffer containing 190mM glycine, 25mM Tris base, 0.05% SDS (w/v) and 20% methanol(v/v). Nitrocellulose paper and Whatman paper were cut to the size of the gel and incubated in the blotting buffer for 10 mins. Using a western blot cassette holder, 4 filter papers were placed on top of the sponges, followed by nitrocellulose paper and the gel. All air bubbles were carefully removed. The second set of filter papers were placed on top of the gel and the cassette closed before transfer to a western blotting tank (Hoeffer Scientific) making sure that the nitrocellulose paper was nearest to the positive electrode (anode). Blotting buffer was added to the tank and the proteins were blotted at 150mA for 2 hours.

After this the cassette was removed from the tank and the gel stained with Coomassie Brilliant Blue for analysis of complete transfer of protein to the nitrocellulose paper. The positions of different molecular weight markers were enhanced on nitrocellulose paper with a pencil (*Appendix*).

3.4.4.1 Blocking buffer

The nitrocellulose was incubated in blocking buffer consisting of 5% (w/v) non-fat dried milk in 0.5% Tween 20/1x PBS (*Appendix*) for 1 hour at room temperature or overnight at + 4°C (*Appendix*).

3.4.4.2 Probing the blots

After a 5-minute wash with 0.5% Tween 20/1x PBS, the blot was then transferred to a 50ml falcon tube containing 5mls of the diluted primary antibody (rabbit anti-bovine xanthine oxidase (Chemicon), 1:1000 dilution in 5% non-fat dried milk in 0.5% Tween 20/ 1x PBS). This was incubated on a roller plate for 1½ hours and thoroughly washed in 0.5% Tween 20/1x PBS 6 times (5mins per wash). The blot was then incubated with the diluted secondary antibody (conjugated horseradish peroxidase swine-anti rabbit (Dako) 1:1000 dilution in 5% non-fat dried milk in 0.5% Tween 20/1x PBS) for 1½ hours. The blot was again washed in 0.5% Tween 20/ PBS 6 times for 5mins each (*Appendix*).

3.4.4.3 Chemiluminescence detection of reaction products

Equal volumes of solution A and solution B from the ECL Amersham detection kit were mixed and added to the membrane making sure the whole of the membrane was covered. Only proteins bound by the primary and secondary antibodies were detected by the utilisation of the hydrogen peroxide/HRP oxidation of luminol. The oxidised luminol emits light at a wavelength of 425nm that is detected by blue light sensitive autoradiography film.

The membrane was incubated for 90 secs and the excess reagents drained off and covered in SaranWrap™ ensuring that all bubbles were smoothed out and placed in a film cassette, protein side up. In a dark room, under safe light conditions, the blots were exposed to Kodak autoradiography film for a range of time points (Kodak Film: Kodak Scientific Imaging Film X-Omat AR (XAR-5) 18 x 24 cm.- Eastman Kodak Co). The films were then processed to reveal the reaction products in an automated processor (Fuji RG II X-ray film processor (Fuji photo film company, Ltd, Japan))(*Appendix*).

3.5 Immunoblotting (Dot blots)

3.5.1 Xanthine oxidoreductase

Once SDS-PAGE and Western Blotting established the characteristics of XOR protein, an immunoblot technique was also utilized for rapid screening for XOR protein in aortic samples. This technique relies on the ability to transfer a known quantity of protein (negative charge) to a nitrocellulose membrane (positive charge) using suction alone. The proteins are not separated according to weight as with a SDS/PAGE. The membrane is then probed with the relevant antibodies and the product detected using an identical technique as for Western blotting. Radiographic films can be digitally imaged and the density of each sample analysed using imaging software. The optical density of each well (sample) can then be measured and compared in a semi-quantitative manner.

One piece of soaked filter paper, followed by nitrocellulose paper was place in a Jencons dot blot tank, and the lid tightly applied. Samples were loaded (20µl of 1 µg/µl) into each well, taking care to avoid air bubbles. Suction was applied for approximately 20-30 seconds, after which the nitrocellulose paper was removed from the tank and stained with Amido black to confirm equal and complete transfer of protein to the nitrocellulose paper.

An identical procedure as for Western blotting was used to probe the blots to detect the presence of XOR protein. Purified bovine XOR was used as a positive control. Each analysis was performed in triplicate. Radiographic films were digitally imaged and stored as Tagged Image Format File (TIFF files). The mean pixel intensity at each well

position was measured using The Scion Corporation Imaging program (Version 4.0.2; Web site: www.scioncorp.com). A constant area (23.75mm^2) was measured for each sample.

All results were obtained as mean pixel intensities measured in a constant area of the blot (fixed area equivalent to the area covered by the wells; 23.75 mm^2). A blank reading was obtained and this value was subtracted from all standard and test sample readings. Relative densitometric of control tissue and aneurysm tissue were compared.

3.5.2 Nitrotyrosine immunoblotting (3 NT)

An identical technique as described for XOR immunoblotting was used to quantify the relative amounts of 3 NT within aortic tissue. Twenty micrograms of total protein was loaded per well. Anti-nitrotyrosine (Upstate Biotechnology) was used at a dilution of 1:1000. The sections were washed and incubated with a conjugated horseradish peroxidase swine-anti rabbit secondary antibody, 1 in 2000 dilution (Dako).

Each analysis was performed in triplicate. Radiographic films were digitally imaged and stored as TIFF files. The mean pixel intensity at each well position was measured in an identical method as **Section 3.5.1** (*Appendix*)

3.6 Enzyme linked immunosorbent assay (ELISA)

3.6.1 Sample preparation

An Enzyme linked immunosorbent assay (ELISA) technique was used to quantify XOR protein in tissue homogenates. A sample of aortic tissue was placed in Hank's balanced salt solution (HBSS) and transferred to the laboratory. Samples were prepared by homogenising 100 mg wet weight of aortic tissue in 1ml of ELISA buffer solution. Specimens were centrifuged at 1000 rpm and stored at - 70 ^{0}C. Following rapid thawing, specimens were further centrifuged at 5,000 rpm and the supernatant retained for subsequent experimentation. Total protein content was determined by the Bradford assay (*Appendix*).

3.6.2 Plate preparation

Immulon 1B (Dynatech Lab) microtitre plates were coated with 100µl 2ug/ml anti-XO IgM antibody (1:500 dilution, Neomarkers Mab 3) in 0.05 M carbonate buffer, pH 9.6 and incubated overnight at 4^{o}C.

Following three washes with 1x PBS, the plates were coated with 200µl Tween 0.05%/1% BSA for 2 hours at 37 ^{o}C. After an additional three washes with PBS/Tween, a sample volume of 100µl, including positive controls (Bovine XO 2-4µg/ml) and

blanks in PBS/Tween/0.1% SDS were added. Samples were serially half diluted from 1: 2.5 to 1: 20. All wells were incubated overnight at 4°C.

Following three washes with PBS/Tween, 100µl of biotinylated anti-XOR chicken antibody (1:1000) was then added, and incubated for 2 hours at 37 °C. After a further three washes with PBS/Tween, a streptavidin-peroxidase conjugate (1µg/ml, 1:500 dilution, Jackson Labs) was added and incubated for 20 minutes at room temperature followed by three washes with PBS/Tween (*Appendix*).

3.6.3 Detection of product

One hundred microlitres (100µl) of orthophenylenediamine (OPD) in citrate/phosphate buffer + H_2O_2 was added to develop a colour. The colorimetric reaction was stopped with 25µl 4M H_2SO_4. The plates were read at 492 nm and 405nm (Background) in a Dynex MRX-II Microplate Reader (Dynex Technologies). XOR concentration was adjusted for total protein and expressed as XOR ng/mg total protein. Each sample was analysed in triplicate (*Appendix*).

3.7 Determination of XOR activity

3.7.1 Sample preparation

Samples were prepared as described in **Section 3.4.1** and total protein estimated using the Bradford assay.

3.7.2 Flurometric measurement of XOR activity (Pterin oxidation)

Xanthine dehydrogenase has a relatively low specificity for its reducing substrates and will oxidise pteridines in addition to many purines. The oxidation of pterin (2-amino-4-hydroxypteridine) to the fluorescent product isoxanthopterin provides a sensitive assay for XOR.

Xanthine oxidase enzyme activity in aneurysm homogenates was measured using a fluorometric assay monitoring the conversion of pterin (a xanthine substitute) to the fluorescent reaction product isoxanthopterin (*Beckman et al. 1989*). XO was assayed in the presence of pterin only, with molecular oxygen serving as an electron acceptor. Combined XO and XD activity was assayed in the presence of methylene blue as the electron acceptor. The samples were warmed to 37 °C in a water bath prior to testing. An aliquot of homogenisation supernatant (50µl) was diluted in 940µl H_20 and placed in a quartz cuvette in a fluorimeter. Measurements were performed using 5 nm bandwidth slits and excitation/emission wavelengths of 345 and 390 nm, respectively. The rate of pterin oxidation was determined after adding 10 µl of 10mM pterin (final conc 100 µM). Once a linear rate had been measured, 10 µl of 10mM methylene blue

was added as an electron acceptor to measure the combined activities of XO and XDH (Total XOR activity). Methylene blue was used as an electron acceptor in place of NAD+ because the fluorescence of NADH, the reaction product overlaps with that of isoxanthopterin. The reaction was then inhibited by the addition of 10µl of 10mM Allopurinol, and a known final concentration of isoxanthopterin was added (10µl of 100 µM; final conc 1µM) as an internal standard. The immediate fluorescence increase after addition of isoxanthopterin provides an internal standard for calculating enzyme activity.

The units of activity may be calculated by the following equation:

Activity (pmoles/min) = (change in fluorescence with pterin/100%Isoxanthopterin) x1000

Activity was corrected for protein content and expressed as pmoles/min/mg protein.

3.7.3 Lucigenin-enhanced chemiluminescence

Xanthine oxidase generates superoxide radicals using xanthine or NADH as substrates with oxygen as the terminal electron acceptor. The generation of superoxide radical was measured using the oxidation of lucigenin. Superoxide radical reacts with lucigenin, to yield an unstable dioxetane that spontaneously decomposes to produce acridone, which then also decomposes to the ground state. This final decomposition emits light which can then be detected using a photomultiplier tube (PMT) (*Faulkner et al. 1993*). Experiments were completed using a 96-well plate-reading chemiluminometer (Anthos, Lucy 1). A green glass filter of 492nm (GG492nm) was used to remove unwanted non-lucigenin-emitted light. (Peak light emission for lucigenin is 500nm). Assays were performed in triplicate in a final volume of 200µl. Samples were prepared as originally described in **Section 3.4.1.** Samples were centrifuged at 2000 rpm for 10 minutes and supernatants retained for experiments. An aliquot of homogenisation supernatant (50µl) was placed in a well and 10µl of 2mM Diethylenetriaminepentaacetic acid (DTPA) and 40µl PBS were added. Lucigenin was made up to a stock concentration of 2mM in PBS and Hypoxanthine made up to a stock concentration of 1mM in PBS. Each plate was allowed to equilibrate to 37°C by placing the plate into the closed reaction chamber of the chemiluminometer (Lucy) for 5 min. The reactions were initiated by the automated injection of 50µl of Hypoxanthine followed by 50µl of lucigenin into the wells of a 96-well plate. Each well was measured sequentially with an integration time of one second per well. Eight sets of triplicates could be assayed at a time, and forty measurements were made per well. Measurements of the peak height of reaction were made, as this is where the reaction rates were at their maximal.

3.8 Cell culture

3.8.1 Tissue preparation

Aortic wall biopsy specimens (2cm ^{2}) were excised from the anterior aortic wall at the time of aortotomy during operative aneurysm repair or from the aortic cuff of cadaveric organ donors. All tissues were placed in Hanks balanced salt solution (HBSS) and transferred to the labarotory.

3.8.2 Primary culture of arterial explants and monolayer culture of VSMCs

Vascular smooth muscle cell cultures were prepared as described by Patel (*Patel et al. 1996a*). Arterial tissue was placed in a petri dish and washed in fresh HBSS. Atheromatous material was removed. The intimal and adventitial layers were aseptically dissected from medial tissue, which was subsequently diced using a 15 mm scalpel blade into 1mm^3 pieces. Three or four segments were placed per well on a 6 well plate and were cultured as explants in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum, Penicillin (100 IU/ml), Streptomycin (100μg/ml) and L-Glutamine (0.2mM) in an atmosphere of 5% CO_2, 95% air at 37° C. Two ml of media were added per well and was replaced every 2-3 days.

3.8.3 Trypsin/EDTA

When the cells reached 75% confluence the cell populations were disaggregated by a 1x solution of Trypsin/ EDTA (*Appendix*). An equal volume of 10% (v/v) foetal calf serum/DMEM was added and the resulting supernatant with disaggregated cells was placed in 50ml Falcon™ (Farenheit) tube. This solution was then pelleted by centrifugation, 1100 rpm (Bench top centrifuge, Denley) for 10mins. The supernatant was aspirated, leaving just the pellet of cells. This pellet was re-suspended in 5mls of tissue culture medium and transferred to either a T_{25} (25cm^2) filter capped cell culture flask (Falcon™) or another six well plate. This was placed in a 37°C humidified incubator (5%CO_2/95% air) overnight to allow cells to adhere to the plastic. The media was replaced the next day. This represented population II (P2) of vascular smooth muscle cells isolated from aortic media (*Appendix*).

3.8.4 Cell counting

Cells from passage 2 or 3 were trypsinized and seeded into T_{25} culture flasks at a known density of 2.5 x 10^5 cells per flask (10,000/cm^2) or a 6 well plate at a density of 100,000 cells per well (14,200/ cm^2) for experimentation. Cells were counted using a Bright line hemocytometer (Sigma). When the cells reached 75% confluence the cell populations were disaggregated by a 1x solution of Trypsin/ EDTA. Following re-suspension, a

small amount of cell suspension was transferred to the chamber of the hemocytometer. Each chamber was allowed to fill by capillary action and care was taken not to overfill or underfill chambers (*Appendix*).

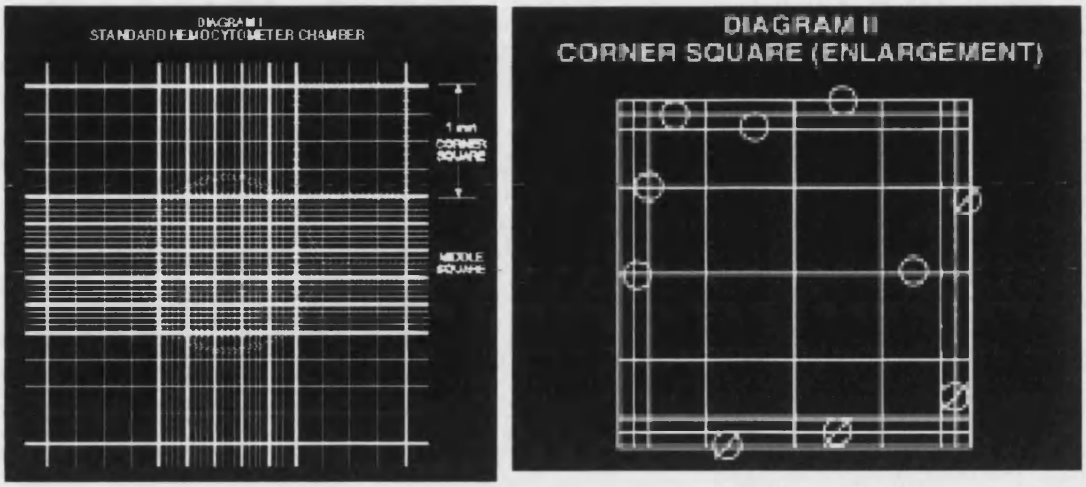

Figure 3-1 Hemocytometer view.

The circle indicates the approximate area covered at 100x microscope magnification. Include cells on top and left touching middle line (o). Do not count cells touching middle line at bottom and right (Ø). Count 4 corner squares and middle square in both chambers. Cells within five 1mm^2 squares were counted. Each square of the hemocytometer, represents a total volume of 0.1 mm^3 or 10-4 cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and total number of cells) were determined using the following calculations;
Cells per ml = the average count per square x 10^4
Total cells = cells per ml x the original volume of fluid from which cell sample was removed.

3.8.5 Cell count and viability assay

Trypan blue is a dye that is not taken up by viable cells with an intact plasma membrane. Non-viable cells take up the dye and appear blue in colour.

100µl aliquots of cell solution were mixed with equal volumes of 0.4% Trypan blue solution (Sigma) and allowed to stand for 5 minutes at room temperature.

A 10µl aliquot of this solution was added to the haemocytometer by capillary action. Cells were viewed by low power transmitted light microscopy and cells within the central 0.1mm^3 section of the haemocytometer were counted.

Total cell counts were calculated by counting all the cells, blue and clear, in the central area of the two counting chambers and dividing by two and then multiplying by 1x10^4. This figure represents the original cell number before dilution and expressed as cells per ml. Viable counts were calculated by repeating the above procedure by counting only the unstained cells. The percentage viable count was calculated (*Appendix*).

3.8.6 Characterisation of VSMCs

10,000 cells were plated onto an 8-well chamber culture slide (Lab-tek II chamber slide, Nalge Nunc International). The culture slides were placed in a humidified 37°C; 5% CO_2; 95% air and incubated for 24hours. The slides were washed three times in cold 1x PBS (w/o Ca^{2+} and Mg^{2+}) (Gibco), fixed with ice-cold methanol:acetone (1:1)v/v for 3 minutes and washed again three times with 1x PBS.

The slides were incubated in blocking buffer (5% non-fat dried milk, Marvel® in 1x PBS/0.5% Tween 20 (v/v)) for 1hr and further incubated with diluted (1:25) mouse monoclonal anti-α smooth muscle actin alkaline phosphatase conjugated antibody (Sigma) in blocking buffer for 1 hour at room temperature. Control slides were incubated with the blocking buffer alone and an irrelevant alkaline phosphatase conjugated antibody (negative control) in parallel. The cells were washed with 1x PBS/0.5% Tween 20 (v/v) (6x 5 mins) and then finally developed with Sigma Fast Red™ (Fast Red TR/Napthol AS-MX Phosphate (4-chloro-2-methyl-benzenediazonium/3-hydroxy-naphthoic acid, 2,4-di-methylanilide phosphate (a napthol AS-MX) in Tris Buffer (0.1M) at room temperature. The cells were rinsed in cold tap water to stop the reaction, counterstained with Mayer's haematoxylin, rinsed with H_2O and mounted in Aquamount (Merck, Lutterworth, UK). Slides were photographed using a Zeiss photomicroscope mark III (*Appendix*).

3.8.7 XOR immunocytochemistry

In addition, cells seeded onto an 8-well chamber culture slide were probed with an antibody to xanthine oxidase. The cells were incubated in blocking buffer (5% non-fat dried milk, Marvel® in 1x PBS/0.5% Tween 20 (v/v) for 1hr and further incubated with diluted (1:100) rabbit polyclonal anti-bovine XO antibody (Chemicon, Harrow, UK) in blocking buffer overnight at 4°C. Control slides were incubated with the blocking buffer alone (negative control) in parallel. The cells were washed with 1x PBS/0.5% Tween 20 (6x 5 mins) and then incubated with biotinylated goat anti-rabbit IgG (1:100) (Vector Labs, Peterborough, UK.) in blocking buffer for 1hr. After rinsing (6x5mins), the slides were incubated with a 1:100 dilution of an alkaline phosphatase-conjugated avidin biotin complex (ABC) (Vectastain, Vector Labs, Peterborough, UK.) for 30mins. The cells were washed again with PBS/0.5% Tween 20, and developed in identical manner as for α-actin characterisation.

3.9 RNA extraction, reverse transcription and PCR

3.9.1 RNA and protein extraction

RNA and protein were isolated from cell culture experiments using RNA STAT 60, a third generation reagent for the isolation of RNA (AMS Biotechnology). The manufacturers' protocol was followed. All items of equipment used for RNA isolation and subsequent experimentation were either pre-treated with Diethyl pyrocarbonate (DEPC 0.1% v/v, Sigma) autoclaved and dried overnight at 120°C, or were certified to be RNAse and DNAse free (ABgene).

Cells were lysed directly by the addition of 1ml of RNA STAT 60 per T_{25} flask (40µl/cm^2) or 0.5 ml per well of a 6 well plate (140µl/cm^2). Samples were stored at room temperature for 5 minutes before the addition of 0.2 ml chloroform per 1ml RNA STAT 60 used. Samples were covered and vigorously shaken for 15 seconds then allowed to stand at room temperature for 15 minutes before being centrifuged for 15 minutes at 12,000 g (4°C) (Biofuge fresco). The RNA remains in the aqueous phase and DNA/protein remain in the inter-phase and organic phase (*Appendix*).

3.9.1.1 RNA extraction

The aqueous phase was carefully removed and transferred to a fresh 1.5 ml eppendorf tube to which 0.5 ml of isopropanol per 1 ml RNA STAT 60 was added. The inter-phase and organic phases were retained for subsequent isolation of protein **(Section 3.9.1.3).** Samples were stored at room temperature for 10 minutes before centrifugation for 10 minutes at 12,000 g (4°C). The RNA precipitate was often invisible before centrifugation but formed a gel-like pellet on the side and bottom of the tube following centrifugation.

The overlying liquid was removed and 1 ml of 75% ethanol was added per 1 ml RNA STAT 60 that was initially added. Samples were vortexed and centrifuged at 7,500 g for 5 minutes (4°C), before the RNA pellet was aired dried for 10 minutes. RNA pellets were dissolved in RNAse and DNAse free distilled water (GibcoBRL life technologies) by repetitive pipetting and an incubation for 15 minutes at 55-60°C. Samples were stored at -70°C. Samples were stored for a maximium of 3 months prior to use (*Appendix*).

3.9.1.2 Protein extraction

To the retained inter-phase and organic phase from **Section 3.9.1.1.** 0.3 ml of 100% ethanol per 1 ml RNA STAT 60 was added. Samples were mixed by inversion, stored at

room temperature for 5 minutes and centrifuged at 2000 g for 5 minutes. The phenol-ethanol supernatant was carefully removed and the DNA pellet discarded.

Proteins were precipitated from the phenol-ethanol supernatant by the addition of 1.5 ml of isopropanol. Samples were stored at room temperature for 10 minutes before centrifugation for 10 minutes at 12,000 g (4°C). The supernatant was discarded and the protein pellets retained.

Protein pellets were washed with 2 ml of 0.3 M guanidine hydrochloride in 95% ethanol and then stored at room temperature for 20 minutes before centrifugation at 7500 g for 5 minutes (4°C). A further 2 ml of 100% ethanol were added, followed by further storage at room temperature for 20 minutes before further centrifugation at 7500 g for 5 minutes (4°C). This washing process was repeated 3 times.

Finally the protein pellets were vacuum dried for 15 minutes and then dissolved in 1% SDS (sodium dodecylsulfate) by repetitive pipetting and incubation at 50°C. The solution was further centrifuged at 10,000 g for 10 minutes (4°C), and the supernatant transferred to a fresh tube for subsequent experimentation (*Appendix*).

3.9.2 Quantification of RNA

The concentration of RNA was determined by measuring absorbance at 260 nm. An RNA sample with an A_{260} of 1 has a concentration of 40µg/ml. In practice, absorbance measurements are performed using a 1:100 dilution of the RNA sample prepared in RNAse and DNAse free H_2O.

All measurements were performed using UV Biotech Photometer (Jencons) which was blanked with 200µl of distilled RNAse DNAse free water (Gibco BRL) in a Quartz cuvette. Samples were prepared by dissolving 2µl of total RNA in 198µl of distilled RNAse DNAse free water (Gibco BRL). Measurements were performed at 260 nm and 280 nm. The total amount of RNA is given by the formula

Total RNA = A_{260} x 40 x dilution factor (100)

A measure of the purity of the RNA sample is obtained by the 260/280 ratio. Ideally the ratio should be greater than 1.5.

3.9.3 Reverse transcription

Reverse transcription was performed using ABgene's First Strand Synthesis Kit following the manufacturers protocol. Sterile RNAse free plastics were used at all times. 1-2µg of total RNA was mixed with 1µl of anchored oligo dT (0.5 µg/µl) and the total volume made up to 13µl with sterile RNAse DNAse free water in a thin-walled reaction tube (ABgene). The tubes were placed in a Thermal cycler (Gene E, Techne) and heated at 70°C for 5 minutes to remove any secondary structure and then placed on ice.

Anchored oligo dT primer is designed to anneal at the mRNA/poly-A junction rather than at a random point within the poly-A tail. By eliminating transcription through the poly-A tail, use of this primer provides more effective cDNA synthesis.
The following components were added, mixed well and spun gently; 4µl (5x) First Strand Synthesis buffer, 2µl dNTP mix (5mM each) and 1µl of RTase. Tubes were incubated at 47°C for 30 min followed by 75°C for 10 minutes to inactivate the RTase. Samples were stored at -20°C before proceeding to polymerases chain reaction amplication (PCR)(*Appendix*).

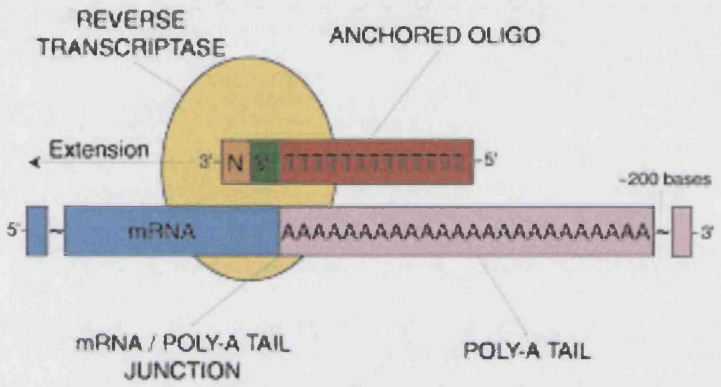

Figure 3-1 Priming cDNA synthesis with anchored oligo dT

The anchored primer is a mixture of primers that all have the basic structure 5'-p (dT)-dV-dN-3' where dV is either dG, dA or dC and dN is any base. The dT bases pair with the 5' region of the poly-A tail, the dV with the final base at the 3' coding region of the mRNA and the dN pairs with the base adjacent to this one, within the mRNA. This anchors the primer to the junction between the poly-A tail and the portion of mRNA to be reversed transcribed.

3.9.4 PCR Amplification

PCR in conjunction with reverse transcription (RT–PCR) was used to study mRNA expression. ABgene®'s ReddyMix™ PCR Master Mix was used as it contains all the components for PCR except template and primers (*Appendix*).

3.9.4.1 XOR PCR

A 288bp fragment of XO cDNA was amplified by PCR using XO specific primers designed according to the rat liver XO cDNA nucleotide sequence (*Nishino et al. 1997*). The primer sequences selected were 5'- AGT ATG TAC ACA CTG CTC CGG – 3' for the forward primer, positioned between nucleotide numbers 364 -385. The reverse primer sequence was the 21 bp oligomer positioned between nucleotide numbers 631-652, thus: 5'- AAG GGG GGT CTC CAA GAC TTC -3'.

2µl of cDNA were mixed with 8.5µl of RNAse DNAse free water, 12.5µl of ABgene®'s ReddyMix™ PCR Master Mix and 1µl each of forward and reverse specific primers. The reagents were mixed in the tube and covered with 30µl of liquid paraffin. Tubes were heated to 94°C for 5 minutes followed by 35 cycles of PCR amplification. Each cycle consisted of 94°C for 45 seconds (denaturing), 58°C for 45 seconds (Annealing) and 72°C for 45 seconds (Elongation). After the last cycle, samples were incubated for a further 7 minutes at 72°C to complete both strands. Samples were then analysed or stored at -20°C (*Appendix*).

3.9.4.2 GAPDH PCR

GAPDH primer sequences (7.5pmoles/µl) obtained from commercially available source (GIBCO BRL)

Forward (5′ to 3′) AAA GGG TCA TCA TCT CTG CC

Reverse (5′ to 3′) TGA CAA AGT GGT CGT TGA GG

Product size 576 base pairs

Tubes were heated to 94°C for 5 minutes followed by 35 cycles of PCR amplification using a thermal cycler. Each cycle consisted of 94°C for 45 seconds (denaturing), 56°C for 45 seconds (Annealing) and 72°C for 45 seconds (Elongation). After the last cycle, samples were incubated for a further 7 minutes at 72°C. Samples were then analysed or stored at -20°C (*Appendix*).

3.9.4.3 MMP-2 and MMP-9 PCR

MMP-2 and MMP-9 PCR was carried out using commercially available primers (R&D Systems, human MMP-2 PCR Primer Pair, RDP-84-025, MMP-9 PCR Primer Pair, RDP-96-025). MMP-2 product size was 449 base pairs (provided internal positive control 380 bp). MMP-9 product size was 564 base pairs, (provided internal positive control 380 bp).

Primer Pairs were reconstituted in 50µl of autoclaved de-ionized water for a final concentration of 7.5µM each primer. One microlitre of cDNA was mixed with 12µl of RNAse DNAse free water, 15µl of ABgene®'s ReddyMix™ PCR Master Mix and 1µl each of forward and reverse specific primers. The reagents were mixed in the tube and covered with 30µl of liquid paraffin to prevent evaporation. Positive Control (Supplied) was resuspended in 30µL of autoclaved deionized water. Tubes were placed in the thermal cycler and heated to 94 °C for 4 minutes followed by 30 cycles of PCR. Each cycle consisted of 94 °C for 45 seconds (denaturing), 55 °C for 45 seconds (annealing) and 72 °C for 45 seconds (Elongation). After the last cycle, samples were incubated at 72 °C for a further 10 minutes (*Appendix*).

3.9.5 PCR product analysis

The amplification products were analysed by agarose gel electrophoresis and visualised using ethidium bromide staining. Agarose gels were prepared by dissolving 1.2 g of Agarose (Promega) in 100 ml of 1x Tris base, Borate, Ethylenediamine tetracetic acid disodium salt (EDTA) (1x TBE). 2µl of ethidium bromide (1mg/ml) were added to give a final concentration of 20ng/ml. Gels were cast in Embi Tec Run One™ Electrophoresis casts and allowed to set at room temperature.

Set casts were transferred to Embi Tec Run One™-Electrophoresis cell and 10µl of PCR product loaded per well. A 100 bp DNA ladder (Promega) together with marker Blue/orange 6x loading dye (Promega) was loaded so that PCR product sizes could be identified. The gel was then subjected to electrophoresis at a constant voltage of 50v and run until the orange dye marker within the DNA ladder well reached the distal marker. Gels were visualised using UV transillumination (Gene Genius) and digital images stored as TIFF files. The mean pixel intensity at each PCR product position was measured using The Scion Corporation Imaging program (Version 4.0.2; Web site: www.scioncorp.com). A constant area (23.75mm^2) was measured for each sample. All results were obtained as mean pixel intensities measured in a constant area of the blot (fixed area equivalent to the area covered by the wells; 23.75 mm^2). A blank reading was obtained and this value was subtracted from all standard and test sample readings. Results are expressed as product/GAPDH ratio (*Appendix*).

3.10 MMP ELISA

Pro-MMP-2 and Pro-MMP-9 were quantitated using a sensitive commercial immunoassay against MMP-2 (Chemicon ECM492) and MMP-9 (Chemicon ECM494) respectively. These immunoassay kits utilize antibodies immobilized on a bead matrix, in combination with enzyme-labelled antibodies, directed against different antigenic sites on the same MMP molecule. Upon addition of an MMP-2 or MMP-9-containing specimen, the result is an MMP-2 or MMP-9 molecule being sandwiched between the solid phase and enzyme labelled antibodies. After removing unbound enzyme-labelled antibody, the bead containing the sandwich is incubated with enzyme substrate and o-phenylenediamine, resulting in the development of colour. The activity of peroxidase enzyme is proportional to the amount of antigen, MMP-2 or MMP-9, so that MMP-2 and MMP-9 concentrations in specimens can be determined from a standard curve. The MMP-2 kit only measures free pro-MMP-2 and pro-MMP-2 complexed with TIMP-2. It does not recognize active MMP-2. The MMP-9 assay system recognizes free pro-MMP-

9, intermediate 83 KDa MMP-9, and MMP-9 in complex with TIMP-1 with the same efficiency. The assay does not recognize active MMP-9 (67 KDa).
Assays were performed according to manufacturers' instructions. MMP standard solutions were prepared by serially diluting the supplied MMP standard. Fifty microlitres of each standard Curve Solution or Specimen were placed in the bottom of a test tube and 300 µl of Enzyme Labelled Antibody Solution added. One anti-MMP-2 or MMP-9 coated bead was placed into each tube and tubes were incubated at room temperature for 1 hour. The reaction was stopped by the addition of 3.0ml of supplied washing solution. The solution was aspirated and beads were washed three times. Following transfer of each washed bead into a clean fresh tube, 300µL of colouring solution was added and tubes were again incubated at room temperature for 1 hour. Reactions were stopped by the addition of 1.5 ml of Stop Solution. Absorbance at 490 nm was measured.

3.11 Elastolytic activity assays

3.11.1 Succinyl trialanyl 4-nitroanilide (SAAANA) assay

Elastolytic activity secreted into media samples during culture of VSMC monolayers was assayed by degradation of the synthetic tripeptide substrate succinyl trialanyl 4-nitroanilide (SAAANA) by using the release of 4-nitro-anilide chromophore as an index of enzymatic activity as described by Bieth and modified by Patel (*Bieth et al. 1974; Patel et al. 1996a*). SAANA is a sensitive tripeptide substrate cleaved by elastase and related elastolytic enzymes. It is also specific because other non-elastolytic proteases do not degrade it.
Media samples from VSMC cultures (0.1 ml) were added to 50mmol/L Tris-hydrochloride buffer containing 10mmol/L Calcium Chloride (0.2 ml) in triplicate wells of 96 well flat-bottom microtitre plates. An aliquot of SAAANA (10mmol/L, 10 µl) in dimethyl sulphoxide (DMSO) was added per well to initiate the reaction, and the plate was sealed and incubated at 37° C for 48 hours. Porcine pancreatic elastase (PPE) was used as a positive control, and serially diluted to obtain a standard curve. A standard plot was constructed using the corresponding OD values and the PPE concentrations. Absorbance values (A405 nm) were measured by an automatic plate reader (Dynex MRX-II Microplate Reader). Elastase activity was expressed as µg/ml of PPE. The amount of elastase activity in the unknown samples was determined by interpolation, reading the concentration of PPE on the standard curve that corresponds to its absorbance. Optimal pH for the degradation of SAAANA by AAA VSMC media samples has been previously determined to be 7.2 (*Patel et al. 1996a*) (*Appendix*).

3.11.2 MMP Gelatinase activity assay kit

The CHEMICON MMP Gelatinase Activity Assay Kit (Chemicon ECM 701) is a commercially available kit to assess gelatinase activity.

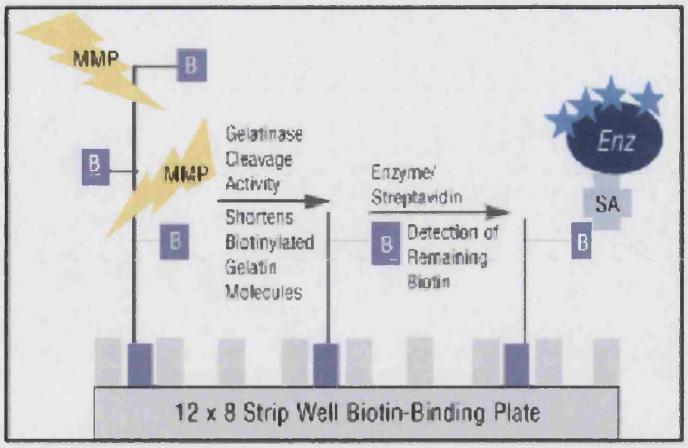

Figure 3-2 Demonstration of the test principle of gelatinase activity assay.

The Activity Assay utilizes a biotinylated gelatinase substrate, which is cleaved by active MMP-2 and MMP-9 enzymes. Remaining biotinylated fragments are then added to a biotin-binding 96-well plate and detected with streptavidin-Enzyme complex. Addition of the enzyme substrate results in a coloured product, detectable by its Optical Density at 450nm (OD_{450}).

Experimental studies using purified, APMA-activated MMP-2 have established the analytical sensitivity of the kit to be less than 5ng/ml. The activity assay kit measures endogenous net MMP activity in solution (it is a product of net elastolysis and inhibitors of elastolysis). APMA (p-Aminophenylmercuric Acetate) activated MMP-2 is supplied as a qualitative positive control for comparison purposes. Quantitative results can be obtained by activating purified human MMP-2 and MMP-9. Endogenous MMP activity or APMA activated activity may be measured.

The assay was carried out according to manufactures instructions. Supplied Pre-activated MMP-2 was used as a positive control. Endogenous MMP activity was measured (Samples were not activated with APMA). Ten microlitres of media samples and 200µl of diluted biotinylated gelatinase substrate were added per well of a 96 well assay plate and allowed to incubate for 2 hours at 37°C. 100µl of this solution were added to a re-hydrated biotin-binding plate and allowed to incubate for 30 minutes at 37 °C. After five washes with (supplied assay buffer), 100µl of a 1:3000 dilution of a supplied streptavidin-enzyme conjugate was added and plates were incubated for 30 minutes at 37 °C. Following a further five washes, 100µl of substrate solution was added

and incubated at room temperature for 20 minutes. Reactions were stopped by the addition of 100µl of stop solution and optical densities were measured at 450 nm in the Dynex MRX-II Microplate Reader. Optical Density values obtained were compared with other test samples to obtain relative activities. Alternatively the MMP control provided with the kit is useful as a positive control for qualitative purposes. Values can be calculated as a percentage of the control ((Positive control OD/Sample OD) x 100 = percent activity)(*Appendix*).

3.11.3 Succinylated Elastin assay

The rationale behind the assay is the use of succinylated elastin as substrate and the detection of exposed primary amines upon enzymatic digestion (*Rao et al. 1997*). Treatment with succinic anhydride blocks free primary amine groups on the side chains of amino acids on the protein (*Hatakeyama et al. 1992*). Primary amines are exposed due to the hydrolysis of peptide bonds in elastin by elastase, which are detected by adding 2, 4, 6-trinitrobenzene sulfonic acid (TNBSA). TNBSA on interaction with primary amines produces a quantifiable colour reaction, the intensity of which is proportional to the number of unblocked amine groups available, which in turn is proportional to elastase activity. Therefore, the colour reaction can be used to measure elastase activity (*Rao et al. 1997*).

Gelatin was succinylated using the procedure described by Rao(*Rao et al. 1997*). Gelatin was dissolved in 50mM sodium borate buffer, pH 8.5, at a concentration of 20mg/ml. An equal amount of succinic anhydride was then gradually added to the solution and the pH of reaction was maintained at 8.0-8.5 by the addition of 1 M NaOH. The succinylated gelatin was then dialyzed extensively against 50mM sodium borate buffer, pH 8.5. As dialysis will result in dilution of the gelatin, the final concentration was determined by Bradford assay.

All assays were performed in a 96 well assay plate. Each reaction contained 200 mg of succinylated gelatin, 50µl of sample made up-to a reaction volume of 150µl with PBS/$CaCl_2$ (1mM) pH 7.2. Blank reactions without substrate but with appropriate buffers and enzyme amounts were performed for each enzyme assay. The reactions were carried out at 37°C for 30 min. Fifty microliters of 0.03% solution of TNBSA was then added to the reaction mixture and allowed to incubate at room temperature for 20min. The optical density (OD) of each reaction was determined at 450nm in the Dynex MRX-II Microplate Reader (*Appendix*).

EXPERIMENTAL SECTION

Chapter 4 Xanthine Oxidoreductase and aortic aneurysms

4.1 Introduction

Xanthine Oxidoreductase (XOR) has been attributed roles in vascular pathology primarily because of its capacity to generate reactive oxygen and nitrogen species (RONS) (*McCord 1985; Millar et al. 1997; Godber et al. 2000b*). The amount and activity of XOR is species, tissue and cell specific, but small amounts of XOR can be detected in various organs with the highest levels of catalytically active enzyme being observed in the liver, intestine, lung and heart (*Terao et al. 1997; Pritsos 2000*). The demonstration of XOR in microvascular endothelial cells and the fact that XOR has been shown to be released into the systemic circulation from the liver and intestine during reperfusion injury may account for the extremely low tissue activity seen when relatively large pieces of tissue are homogenized for enzymatic activity or blotting (*Vickers et al. 1998*).

Levels of both XOR and anti-XOR antibodies are known to be elevated in plasma of atherosclerotic patients and the presence of the enzyme has been demonstrated in vessel walls and atherosclerotic plaques (*Harrison et al. 1990; Swain et al. 1995; Mohacsi et al. 1996; Patetsios et al. 1996; Patetsios et al. 2001*). Swain demonstrated that ferroxidase activity attributable to XOR was present in both carotid endarterectomy plaques and AAA tissue (*Swain et al. 1995*). Patetsios demonstrated using high performance liquid chromatography (HPLC) that both AAA tissue and carotid endarterectomy plaques contained significantly more uric acid (the end-product of XOR activity) than non-atherosclerotic arteries obtained from young cadaveric organ donors. Immunohistochemistry localised XOR to the intima, especially in atherosclerotic plaques and inner media of the artery wall (*Patetsios et al. 1996; Patetsios et al. 2001*). The suggestion from this study was that increased XOR expression/activity resulted in the production of uric acid with the resultant generation of free radicals that promoted vascular injury. There are no published reports suggesting a role of XOR in AAA pathology or quantifying either the amount of XOR protein or XOR activity in AAA tissue compared to aortic tissue from either AOD or normal controls.

4.2 Aims

The aims of this study were;

1. To identify and localise XOR protein/enzyme in the aortic wall using immunohistochemistry and Western blotting techniques
2. To quantify the amount of XOR protein in aortic tissue using an immunoblot and an ELISA assay.
3. To measure XOR activity in tissue homogenates.

AAA tissue was compared to non-AAA derived aortic tissue.

4.3 Patients and Methods

4.3.1 Identification and localisation of XOR in aortic wall

Aortic wall biopsy specimens ($2cm^2$) were excised as described in Section 3.2. Specimens were either stored in Hank's balanced salt solution (HBSS) or Formalin (immunohistochemistry).

4.3.1.1 Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting for the identification of XOR protein

Samples were prepatred as described in **Section 3.4.1**. Approximately 100 mg (wet weight) of aortic tissue was homogenized in 1.5 ml of homogenisation buffer and centrifuged at 10,000 rpm, for 10mins at + 4°C and the supernatant retained (*Beckman et al. 1989*). Total protein was estimated using the Bio-Rad Bradford Protein Assay. XOR protein within aortic samples was detected using SDS-PAGE and an antibody raised against bovine buttermilk XOR as described in **Section 3.4.3 and 3.4.4**. Purified bovine XOR was used as a positive control (Biozyme). Blots were probed a rabbit anti-bovine xanthine oxidase (Chemicon, 1: 500 dilution) and a conjugated horseradish peroxidase swine-anti rabbit secondary antibody (Dako, 1:1000 dilution). Proteins binding the primary and secondary antibody were detected by Chemiluminescence as described in **Section 3.4.4.3**.

4.3.1.2 Localisation of XOR and in human aortic wall sections

Sections were processed for XOR protein using the Vectastain ABC-Alkaline Phosphatase Kit for detecting rabbit immunoglobulin G **(Section 3.3.3)**. Sections probed with a polyclonal rabbit anti-goat XOR (Chemicon, 1:50 – 1:200 dilution) and a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Vectastatin® 1:200 dilution) as described in **Section 3.3.3**.

4.3.2 Quantification of XOR protein in aortic tissue

4.3.2.1 Immunoblotting (Dot blots)

Once SDS-PAGE and Western Blotting established the characteristics of XOR protein, an Immunoblot technique was also utilized for rapid screening of XOR protein in aortic samples **(Section 3.5.1)**. Samples were loaded (20µl of 1 µg/µl) into each well and suction was applied for 30 seconds. Staining with Amido black confirmed equal and complete transfer of protein to the nitrocellulose paper.

An identical procedure as for Western blotting was used to probe the blots to detect the presence of XOR protein. Purified bovine XOR was used as a positive control.

Each analysis was performed in triplicate. Results were analysed as described in **Section 3.5.1.** The parameters of image analysis were kept constant to avoid inter-observer variations. Results are expressed in densitometric units. Each sample was analysed in triplicate and the whole assay repeated three times.

4.3.2.2 Enzyme linked immunosorbent assay (ELISA) for the detection of XOR in aortic tissue

An Enzyme linked immunosorbent assay (ELISA) technique was used to quantify XOR protein in tissue homogenates as described in **Section 3.6**. Samples were prepared (**Section 3.6.1**) and total protein content determined by the Bradford assay (**Section 3.4.2**).

XOR protein immobilised with an anti-XO IgM antibody (Neomarkers Mab 3, 1:500 dilution) was detected with a biotinylated anti-XOR chicken antibody (1:1000) and a streptavidin-peroxidase conjugate (Jackson Labs, 1μg/ml, 1: 500 dilution) and orthophenylenediamine (OPD). Each sample was analysed in triplicate and the assay repeated twice. XOR concentration was adjusted for total protein and expressed as XOR ng/mg total protein (**Section 3.6**).

4.3.3 Determination of XOR activity

Samples were prepared as described in **Section 3.4.1** and total protein estimated using the Bio-Rad Bradford Protein Assay (**Section 3.4.2**).

4.3.3.1 Flurometric measurement of XOR activity (Pterin oxidation)

Xanthine oxidase enzyme activity in aneurysm homogenates was measured using a fluorometric assay monitoring the conversion of pterin (a xanthine substitute) to the fluorescent reaction product isoxanthopterin (*Beckman et al. 1989*)(**Section3.7.1**). An aliquot of homogenisation supernatant (50μl) was diluted in 940μL H_20 and placed in a quartz cuvette in a fluorimeter. The rate of pterin oxidation was determined after adding 10 μl of 10mM pterin (final conc 100 μM). Once a linear rate had been measured, 10 μl of 10mM methylene blue was added as an electron acceptor to measure the combined activities of XO and XDH (Total XOR activity). The reaction was then inhibited by the addition of 10 μl of 10mM Allopurinol, and a known final concentration of isoxanthopterin was added (10 μl of 100 μM; final conc 1 μM) as an internal standard. Activity was corrected for protein content and expressed as pmoles/min/mg protein. Each sample was assayed in triplicate and repeated three times. Bovine XOR (Biozyme) was used as a positive control.

4.3.3.2 Lucigenin-enhanced chemiluminescence

XOR generates superoxide radicals using xanthine or NADH as substrates with oxygen as the terminal electron acceptor. The generation of superoxide radical was measured using the oxidation of lucigenin (**Section 3.7.3**). Assays were performed in triplicate in a final volume of 200µl. Samples were prepared as for the Pterin assay. An aliquot of homogenisation supernatant (50µl) was placed in a well and 10µl of 2mM Diethylenetriaminepentaacetic acid (DTPA) and 40µl PBS were added. Lucigenin was made up to a stock concentration of 2mM in PBS and Hypoxanthine made up to a stock concentration of 1mM in PBS. The reactions were initiated by the automated injection of 50µl of Hypoxanthine followed by 50µl of lucigenin into the wells of a 96-well plate. Each sample was analysed in triplicate and repeated three times. The results are expressed in arbitrary light units (ALU).

4.3.4 Control group, Power calculation and statistical analysis

4.3.4.1 Controls

AAA tissue was compared to non-AAA derived aortic tissue (Controls). Ideally aortic tissue from age and sex matched patients with AOD and normal non-atherosclerotic aorta should have been used for this study. However, this proved to be impossible for several reasons (see discussion **Section 4.5**). In the end, the best alternative for 'control' aortic tissue was the use of cadaveric organ donors and aortic tissue from the cuff on the renal artery. This has been used as control tissue in previous studies (*Patel et al. 1996a*)

4.3.4.2 Power calculations and sample size

No formal power calculation could be undertaken to calculate the number of samples required in either the AAA group or control group. In a previous published study, no statistical difference in XOR activity (using uric acid formation from Hypoxanthine) was seen between endarterectomy and AAA specimens, however, only 8 samples were analysed in each group (*Swain et al. 1995*). In another study, uric acid concentrations, the product of XOR activity, were significantly increased in both AAA and carotid plaques compared to non-atherosclerotic controls. No analysis was undertaken between AAA and carotid plaques in this study, and the data within the paper is insufficient to perform any further analyses (*Patetsios et al. 1996*).

Previous published studies using tissue homogenates that have shown differences between AAA and AOD/normals, particularly with reference to MMPs expression/activity have all included small numbers of control samples, ranging

between 3 and 10 (*Vine et al. 1991; Thompson et al. 1995; Patel et al. 1996a*). In view of this, the aim was to obtain 10 'normal' non-AAA tissue samples.

4.3.4.3 Data presentation and statistical analysis

Statistical analysis was performed using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Data were assessed for deviations from Gaussian distribution using the Kolmogorov-Smirnov test. Continuous variables were expressed as either means ± standard deviation (SD) or Standard error of the mean (SEM) for parametric data. Variables were analysed with students' T test. Statistical significance was considered for p values less than 0.05.

4.4 Results

4.4.1 Patient demographics

The demographics of the two groups of patients used in this study are given in **Table 4.1.** No difference in gender distribution was noted but a statistically significant difference was seen with regard to age (Students t test, $p< 0.001$)

	AAA (n=30)	Control (n=18)	P
Age, y	74 (range 57-89)	47 (range 17-69)	P< 0.001
Gender, % male	84	65	P = 0.11

Table 4-1 Demographic data of patients used in the study of XOR in aortic tissue

4.4.2 Identification and localisation of XOR in aortic wall

4.4.2.1 SDS-PAGE and Western blotting

Fig 4.1 shows a representative blot of human aortic aneurysm tissue depicting a band at the estimated weight of 150 kDa detected using a rabbit polyclonal XO antibody (Chemicon). 30μg of total protein were loaded per lane. Blots were probed with a primary rabbit anti-bovine xanthine oxidase antibody (Chemicon, 1:500 dilution) and a secondary conjugated horseradish peroxidase swine-anti rabbit antibody (Dako, 1:1000 dilution). A characteristic 150 kDa band was only present in a few samples, whereas most samples displayed degradative product with a prominent band at approximately 60 kDa.

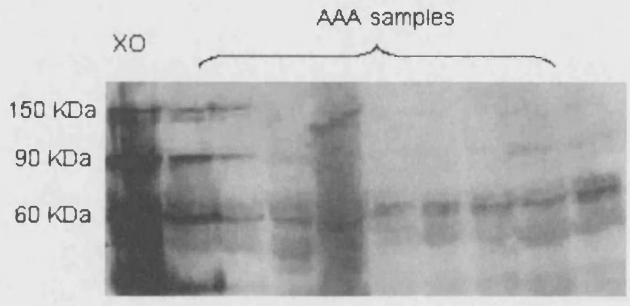

Figure 4-1 Western blot of human aortic aneurysm XOR after SDS-PAGE

4.4.2.2 Localisation of XOR and in human aortic wall sections (Xanthine oxidase immunohistochemistry)

Figures 4.2 and 4.3 indicate human aortic tissue isolated from patients undergoing elective surgery or organ donation showing positive immunoreactivity for XOR. To visualise all cells, including non-immunostained cells, a haematoxylin counterstain was applied which depicts a blue nucleus. Antibody staining was performed on 20 AAA specimens and 8 normal human aortas obtained from young organ donors. Control slides (primary antibody omitted) were negative for any XOR.

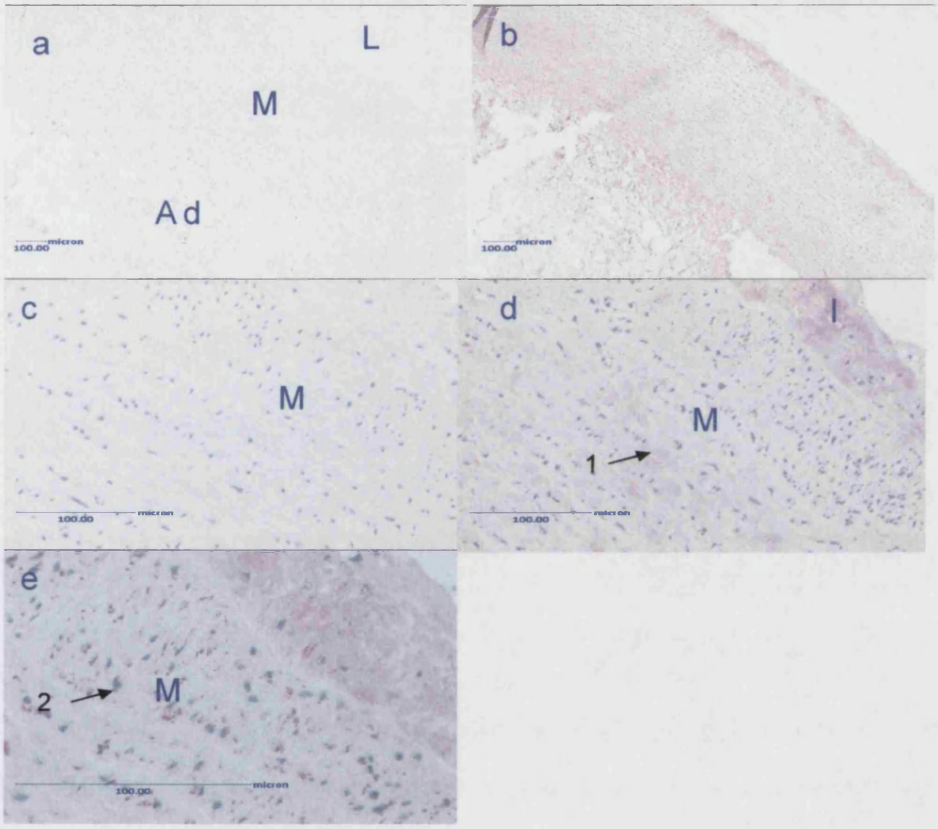

Figure 4-2 Photomicrographs showing the immunolocalisation of XOR (red) in human non-AAA tissue

Specimens were probed with a rabbit anti-bovine XOR, (Chemicon, 1:100). Slides a and c are negative controls. Slide b (x50) shows diffuse positivity for XOR throughout the artery wall. Image d (x 200) and image e (x 400) show both extracellular (1) and intracellular (2) positivity for XOR. *(L=Lumen, M=Media, Ad=Adventitia)*

Control non-AAA tissue (**Fig 4.2**) stained positive for XOR and was localised throughout the artery wall with both extracellular and intracellular positivity for XOR. An almost identical pattern of XOR positivity was visualised in AAA tissue.

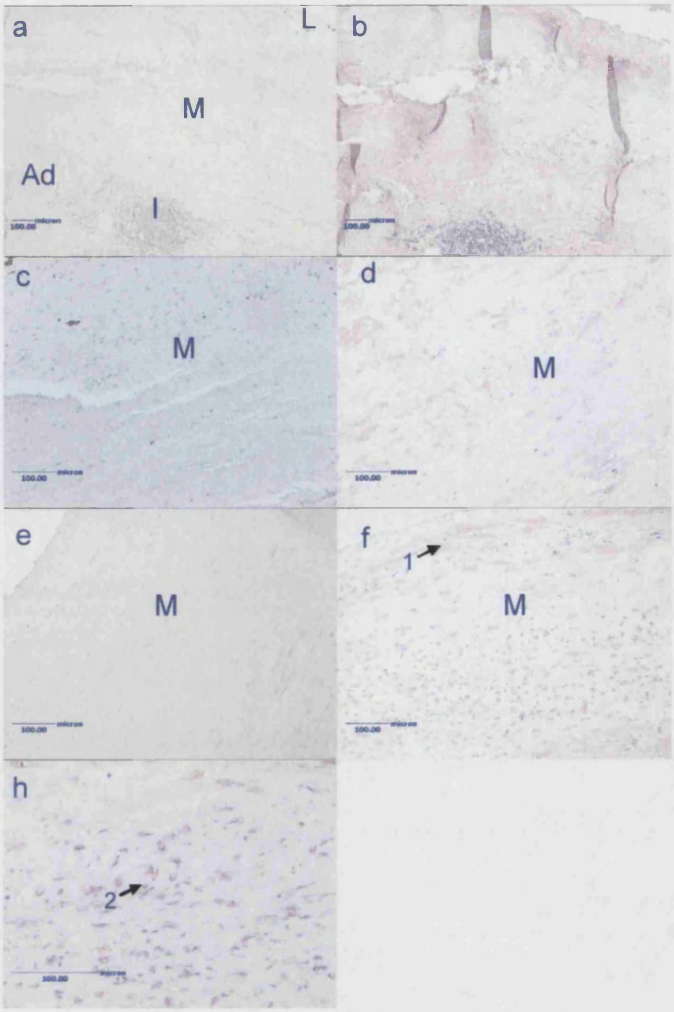

Figure 4-3 Representative Photomicrographs showing the immunolocalisation of XOR (red) in human AAA tissue

Images a, c and e are negative controls. Images b (x50), d (x100) f (x200) and h (x400) show diffuse positivity throughout the aortic wall. Extracellular (1) and intracellular (2) staining is seen. *(L=Lumen, M=Media, Ad=Adventitia, I=Inflammatory infiltrate)*

4.4.3 Quantification of XOR protein in aortic tissue

4.4.3.1 Immunoblotting

The amount of XOR protein in AAA samples was compared to XOR levels in non-aneurysm control tissue using a semi-quantitative immunoblot technique. A positive control using bovine XOR (Biozyme) was used. Blank wells were loaded with 1x PBS. The mean valve of triplicates for each sample was used for each analysis. The assay was repeated twice. These data represent the mean value of two separate analyses (AAA n = 17, Controls n =14). Results are expressed in relative densitometric units.

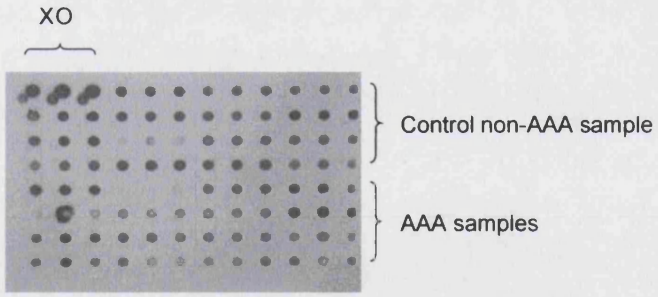

Figure 4-4 Immunodot blot of AAA and control aortic tissue

Samples analysed in triplicate (AAA n =17, control n =14). 20µg of total protein per well. Bovine XOR enzyme used as positive control (20 µg/well). Blots probed with rabbit anti-XOR (Chemicon 1:1000 dilution) and conjugated horseradish peroxidase swine-anti rabbit (Dako, 1:1000 dilution).

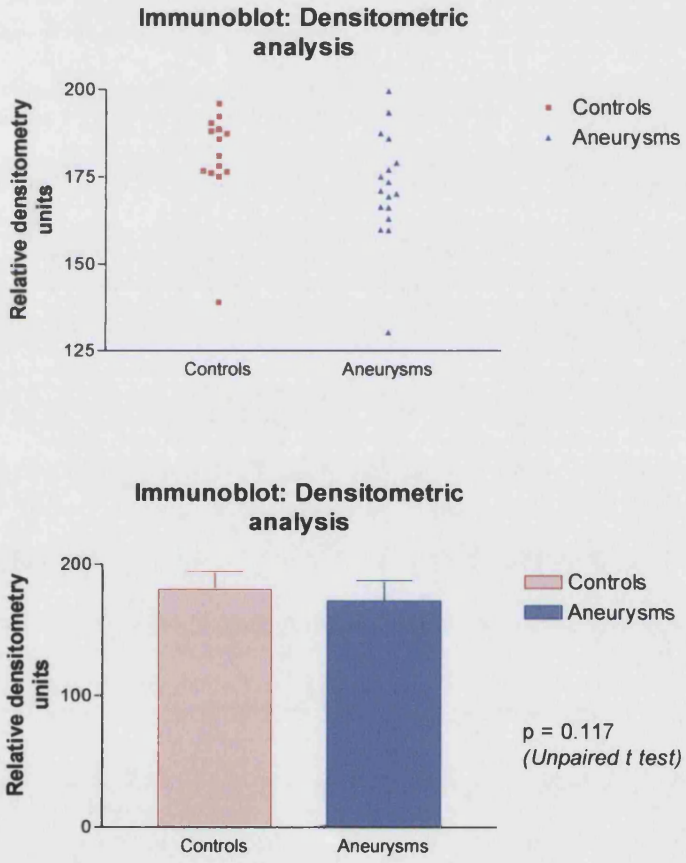

Figure 4-5 XOR protein levels in aortic tissue

Results expressed as relative densitometry units in AAA (n=17) and control samples (n=14) quantified using an immunoblot. Data are mean ± SD of sample triplicates analysed twice.

There was no statistical difference in the levels of XOR protein between AAA and control aortic tissue using immunoblotting.

4.4.3.2 Enzyme linked immunosorbent assay (ELISA) for the detection of XOR in aortic tissue

Each sample was analysed in triplicate and the mean of each sample triplicate used in statistical analysis. The assay was repeated twice. These data represent the mean value of two separate analyses (AAA n = 17, Controls n =12). XOR protein content is expressed as ng/mg total protein.

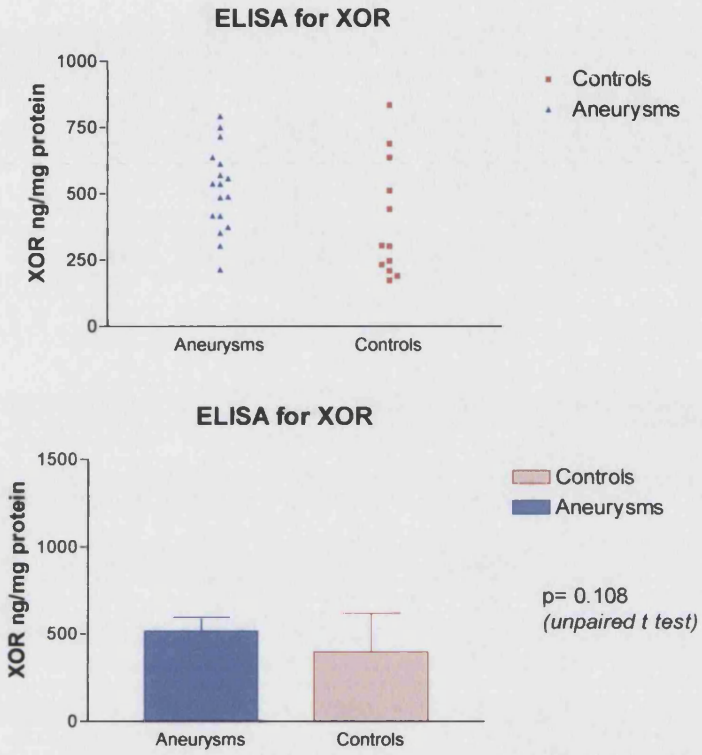

Figure 4-6 XOR protein levels (ng/mg total protein) in AAA (n=17) and control samples (n=12) quantified using an ELISA.

There was no statistical difference in the amount of XOR per mg total protein between AAA tissue and normal controls.

4.4.4 Determination of XOR activity

4.4.4.1 Flurometric measurement of XOR activity (Pterin oxidation)

Due to difficulties in establishing clear differences in the XOR immunoexpression in control and AAA tissue, a fluorometric assay was used to determine whether the the activity of XOR was different in AAA and non AAA tissue. Ten nanograms (10ng) of bovine XOR (Biozyme) was used as a positive control for XOR activity using the fluorometric assay. The experimental blank consisted of distilled H_2O and 100 μM pterin alone. **Figure 4.7** shows the typical reaction rate of pterin oxidation with bovine XOR (Biozyme). The rate of reaction of the oxidase form of XOR (XO) was established by the rate of fluoresence change in the presence of oxygen as the terminal electron

acceptor. The addition of 100μM methylene blue measured the combined activities of XO and XDH (Total XOR activity).

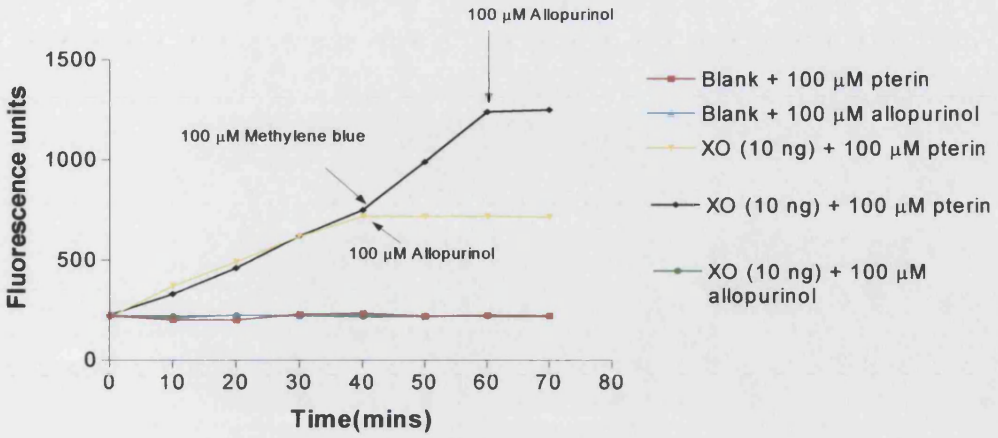

Figure 4-7 Pterin oxidation graph

A typical graph, showing the rate of XOR-catalysed conversion of pterin to its fluorometric reaction product isoxanthopterin.

Figure 4.8 shows the XOR activity detected in AAA and control tissue determined by the fluorometric assay described above. Each sample was analysed in triplicate and the assay repeated three times. These data presented represent the mean values of the three analyses (AAA n = 12, Controls n =10). No measurable XOR activity could be detected in either AAA or control tissue using this assay.

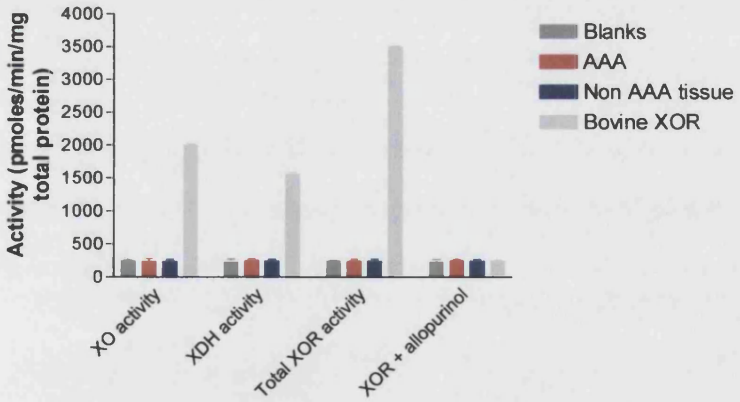

Figure 4-8 XOR activity in human aortic tissue detected using the pterin assay.

4.4.4.2 Lucigenin-enhanced chemiluminescence

Hypoxanthine was used as the assay substrate and ten micrograms (50 μl of 0.2 mg/ml) of bovine XOR (Biozyme) was used as a positive control. Reaction blanks contained 1x PBS. Blank recordings were subtracted from sample values. Each sample was analysed in triplicate and the assay repeated on three occasions. The data represent the mean of three assays. Results are expressed in arbitrary light units per milligram of total protein

(ALU/mg total protein). **Figure 4.9** shows that AAA and control samples contained no measurable activity using hypoxanthine as a substrate.

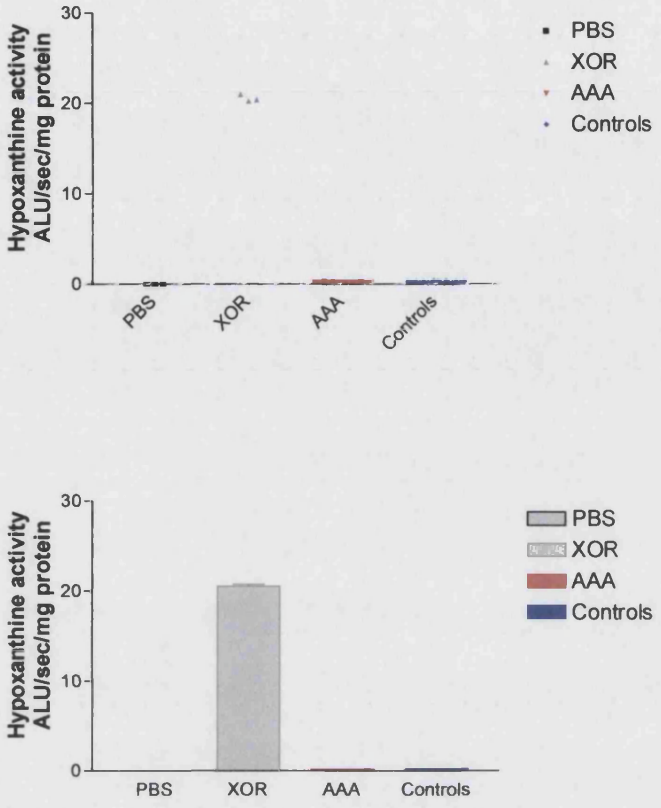

Figure 4-9 Chemiluminesence activity detected in aortic tissue using hypoxanthine as substrate

AAA (n=20) or control tissue (n=12)

4.5 Discussion

The data presented in this chapter demonstrate that XOR is detected in aortic tissue and localises predominantly within the intima and inner media. There is no difference in the level of XOR between AAA and non-AAA control aortic tissue and in addition the levels of XOR activity within aortic tissue are below detectable limits.

Ideally aortic tissue from age and sex matched patients with AOD and normal non-atherosclerotic aorta should have been used for this study. However, this proved to be impossible for several reasons. Firstly, the number of procedures undertaken for aortic occlusive disease (AOD) was significantly less than for AAA disease during this study period and the availability of tissue was limited. Secondly, it is impossible to obtain 'normal aortic tissue' from age or sex matched controls, as these patients do not usually require any aortic procedure. In addition it is worth pointing out that this study was undertaken during a period of intense and unfavourable media attention to medicine and the use of human material for medical research in particular, thus it was difficult not only in obtaining ethical approval but also informed consent. Atherosclerotic material from other arteries was not thought to be a valid control as it could not be argued that the femoral artery had the same biological behaviour as the abdominal aorta.

In the end, the best alternative for 'control' aortic tissue was the use of cadaveric organ donors and aortic tissue from the cuff on the renal artery. This has been used as control tissue in previous studies (*Patel et al. 1996a*). Whilst this is not perfect it was the best option available, however I am aware that conclusions from this work are weakened by the fact that age and sex matched controls were not possible.

The use of end-stage whole tissue homogenates in the research of the pathogenesis of AAAs has potential weaknesses but has been used in the past (*Busuttil et al. 1980; Cohen et al. 1988*). However several important points must be highlighted when drawing conclusions from these types of studies;

1. These studies are unable to separate primary aetiological events from secondary effects, i.e they are unable to distinguish between cause and effect.
2. The presence of an inflammatory infiltrate and altered cytokinetic profile within AAA tissue confounds the issue and prevents meaningful conclusions from being drawn regarding enzyme status or ECM metabolism in the stages of aneurysm formation that precede dilatation
3. The absence of a significant result does not preclude an active involvement of the subject enzyme/protein at some time during the pathogenesis of AAAs. In relation to AAAs, only the two extremes of the condition are being assessed i.e. the non-disease

state (control) and end-stage-disease, the dynamic stage of aneurysm formation is not assessed, and as such no inference can be made about this stage of AAA development.

In this study, SDS-PAGE and Western blotting in combination with immunohistochemistry identified and localised XOR to the media and intima which confirms previous reports (*Swain et al. 1995; Patetsios et al. 1996; Patetsios et al. 2001*). The use of a polyclonal rabbit anti-goat XOR antibody is a valid and appropriate antibody to investigate human XOR enzyme as the human XOR amino acid sequence has been shown to have a significant homology ($\approx$ 90%) to that of other mammalian species (*Amaya et al. 1990; Ichida et al. 1993; Sato et al. 1995*). Immunohistochemical analysis demonstrated that XOR staining was prominent along the intimal surface and inner media (i.e. the region of plaque formation) and separate from the inflammatory infiltrate. Both intracellular and extracellular staining was seen in both control and AAA tissue, with endothelial cell and smooth muscle cells staining positive for XOR suggesting that these cell populations are a significant source for this enzyme. The extra-cellular pattern of XOR staining is supported by evidence that XOR has been previously demonstrated to be localised to the outer surface of the endothelial cell plasma membrane, and is also known to bind to glycosaminoglycans, a significant component of the extracellular matrix (*Adachi et al. 1993; Radi et al. 1997; Vickers et al. 1998*). Two theoretical explanations exist to explain this extracellular pattern of XOR staining.

1. XOR is produced intracellularly and then secreted but binds extracellularly to the cell membrane.
2. XOR is released into the circulation from organs rich in XOR such as the liver and intestine and is then possibly internalised in tissue such as the arterial wall via interactions with glycosaminoglycan (*Yokoyama et al. 1990; Radi et al. 1997*).

Previous studies have demonstrated that substantial differences exist in the level of XOR in different sources of mammalian tissue (*Pritsos 2000*). A Post-mortem study from Finland using RT-PCR, demonstrated very low levels of the XOR transcript in heart, brain, lung and kidney tissues. No XOR activity could be detected in these tissues suggesting a very low constitutive expression of XOR. The only exception to this was in liver and intestine where both expression and activity was significantly higher (*Sarnesto et al. 1996; Saksela et al. 1998*). The results from these studies, however, are inconsistent with the results of an earlier study in which relatively high XOR activities were seen in human post-mortem brain and heart tissues, as well as activities in kidney,

skeletal muscle, spleen and adrenal tissues suggesting a possible leakage of enzyme from disintegrating tissue (*Wajner et al. 1989*). In addition, microvascular endothelial cells have been identified as a source as being rich in XOR activity and this may provide the explanation as for the detection of XOR in the majority of mammalian tissue. These XOR-rich but relatively small sub-populations of cells could account for the extremely low levels of enzyme or activity when relatively large pieces of tissue are homogenized for enzymatic activity or blotting.

XOR has a molecular mass of 300 KDa and is composed of two identical 150 KDa subunits. Purified human XOR from breast milk runs as a single band corresponding to approximately 150 KDa on SDS/PAGE (*Harrison 1997; Sanders et al. 1997*). Human XOR principally exists as xanthine dehydrogenase but is converted into xanthine oxidase during extraction or purification procedures by either thiol oxidation or proteolysis (*Nishino et al. 1997*). This conversion readily takes place during isolation procedures unless thiol reducing agents and protease inhibitors are included in isolation buffers. In addition, limited proteolysis of the rat enzyme with trypsin converts the enzyme into a xanthine oxidase type with concomitant cleavage into 20, 40 and 85 KDa fragments (*Amaya et al. 1990*).

In this study, evidence of XOR protein in aortic tissue was difficult to assess as it was difficult to obtain good clear Westerns blots with characteristic 150 KDa bands. Several observations and difficulties encounted are listed below:

1. The sample total protein loading per well/lane was extremely high. In general 30μg of total protein was required per lane in order to visualise any staining.
2. The concentration/dilution of primary antibody was high, generally in the region of 1:250 to 1: 500. Lower dilutions of primary antibody failed to detect evidence of xanthine oxidase. The conclusion from these two observations is that the total amount and concentration of XOR in the aortic tissue studied, i.e. both control and AAA tissue is extremely small.
3. In all Western blots a significant proportion of samples showed degradation fragments rather than the characteristic 150 KDa band. There are two possible explanations to account for these results. The first explanation is that XOR protein does not exist in the samples and any staining observed was non-specific and a false positive result. In response to this possibility samples were also probed with a HRP conjugated anti XO IgG (Biodesign), and a non-specific rabbit anti human immunoglobulin (Dako). The results from these studies confirmed specificity for XOR as the anti-XOR antibody showed an identical pattern of staining for XOR and

the non-immune immunoglobulin showed no staining for any samples. The second explanation of this is that XOR had undergone proteolysis during either the isolation or experimental procedure. Proteolysis is known to occur during isolation of XOR unless thiol reducing agents and protease inhibitors are included in isolation buffers. In these investigations anti-proteases such as Pepstatin A, Antipain, Leupeptin and Aprotinin were included in the homogenisation buffer. In addition Ethylenediaminetetraacetic acid (EDTA) was added to chelate calcium and other heavy metals which are required for the activity of many proteases, and Phenylmethanesulfonyl fluoride (PMSF) was added as it inhibits serine proteases such as trypsin and chymotrypsin and also cysteine proteases. Dithiothreitol (DTT) was added as a thiol reducing agent. Despite the presence of these compounds it was difficult to isolate XOR as a characteristic 150 KDa on SDS/PAGE and Western blotting. It is possible that XOR proteolysis occurred during the period of sample storage in the Hanks buffered saline solution and a suggestion now in retrospect is that either these compounds are added to the Hanks solution or alternative forms of sample storage/processing are undertaken such as snap freezing in liquid nitrogen and then homogenisation.

Xanthine Oxidoreductase activity is known to differ both between mammalian species and within each species depending on which tissue is assessed. Despite the demonstration of XOR in tissues, human XOR activity is known to be low compared to other species (*Parks et al. 1986; de Jong et al. 1990; Sarnesto et al. 1996*). Human XOR purified from breast milk runs as a single 150 KDa band on SDS/PAGE, however the activity towards most reducing substrates is very low, representing only 2-3 % that of purified bovine milk enzyme (*Harrison 1997; Sanders et al. 1997*). This activity may result from an exceptionally low molybdenum content of the human enzyme as this redox centre is the site to which most reducing substrates donate their electrons (*Godber et al. 1997; Harrison 1997*). The exception to this is liver and intestine, in which a high molybdenum centre activity is noted to traditional substrates and the purified enzyme shows specific activity very similar to those of bovine milk and rat liver enzyme (*Sarnesto et al. 1996*). These observations lead Harrison to postulate that human XOR might be tissue specific, with 'high-activity' enzyme in a limited number of tissues (e.g. liver and intestine) and 'low-activity' enzyme, similar to that in breast milk in the rest (*Harrison 1997*).

In this study the two assays used were dependent on the activity at the molybdenum centre of the enzyme. The data demonstrates that no reproducible XOR activity is

present in any aortic tissue. Significant background readings were obtained from all samples. It was believed that this was due to the heterogeneous nature of samples, however despite centrifugation for both longer and at faster speeds no difference was noted. Sample processing through a PD-10 column (Saphadex™ G-25M, Amersham Parmacia Biotech AB) made no difference. Despite increasing the sample volume to a maximum of 200µl, or increasing the substrate (pterin) concentration to a final of 200µM, no activity could be detected.

Possible explanations to account for no XOR activity being present in aortic samples include;

1. This is a true result and there is indeed no XOR activity is present in aortic tissue
2. The amount of XOR activity present in aortic tissue is below the detection limit of the assays employed, i.e. a problem of sensitivity. It is worth pointing out that we are unaware of a more sensitive assay for XOR other than that described by Beckman (*Beckman et al. 1989*). Interestingly Patetios using high-performance liquid chromatography (HPLC) demonstrated significantly increased levels of uric acid, the end-product of XOR function, in the walls of aortic aneurysms compared to non-atherosclerotic control arteries, suggesting that functioning XOR is present in vessel walls. The data also suggest that there is a functional difference of XOR between aneurysm and non-aneurysm normal tissue, however we have been unable to support this finding (*Patetsios et al. 1996*).
3. It is possible that Harrison's postulation may be correct and the XOR present in aortic tissue may be a 'low-activity' enzyme with little molybdenum centre activity. If this is the case then an alternative assay is required to measure activity at the other redox active centres (*Harrison 1997*).
4. The functioning of XOR requires an intact enzyme. Electrons donated at the molybdenum centre are passed to the FAD site and a terminal electron acceptor, NAD in the case of the dehydrogenase enzyme and molecular oxygen in the case of the oxidase form. Data from SDS/PAGE and Western blotting suggests that in our samples very little XOR was intact and this may explain the lack of activity seen in our samples.

This is clearly disappointing but could be the result of the end-stage of aneurysm formation, and perhaps might have been different in the earlier stages of aneurysm formation.

Chapter 5 Hypoxia and role in AAAs

5.1 Introduction

5.1.1 Arterial wall hypoxia

Arteries with a diameter and wall thickness of the aorta contain areas of hypoxia at the junction of the inner one third and outer two thirds of the vessel wall (*Wolinsky et al. 1967; Heughan et al. 1973*). Experimental animal data and mathematical computational models suggest that this normal physiological hypoxia is exacerbated in conditions such as atherosclerosis, diabetes, hypertension, cigarette smoking, ageing and arterial bifurcation, all of which are known to be associated with AAA formation (*Heughan et al. 1973; Crawford et al. 1991; Santilli et al. 1992, 1993; Santilli et al. 1995; Vorp et al. 1996; Santilli et al. 1998; Vorp et al. 1998; Santilli et al. 2000*). Data from animal *in vitro* and *in situ* systems demonstrate that in normal arteries the pO_2 decreases in the inner media to 20 to 50 mm Hg (*Niinikoski et al. 1973; Crawford et al. 1988; Zemplenyi et al. 1989*). In addition the use of the hypoxic probe NITP in a rabbit model of atherosclerosis confirms that hypoxia exists within atheosclerotic plaques and the inner media (*Bjornheden et al. 1999*).

AAA are invariably associated with laminated intraluminal thrombus (ILT) (*Adolph et al. 1997; Vorp et al. 1998*). Vorp hypothesized that ILT forms a mechanical barrier to oxygen diffusion rendering the intima and inner media anoxic (*Vorp et al. 1996; Vorp et al. 1998*). Data from intra-operative pO_2 measurements with a needle polarographic pO_2 electrode indicated that the pO_2 within the AAA wall was significantly less in patients with ILT and was associated with localised areas of neovascularisation and local wall weakness (*Vorp et al. 1998; Vorp et al. 2001*).

5.1.2 Effects of hypoxia

Hypoxia has been shown to alter the expression of growth factors and angiogenic factors such as vascular endothelial growth factor (VEGF), platelet derived endothelial growth factor (PDEGF), endothelin (ET-1), and vasoactive factors such as nitric oxide synthase (NOS) probably via modulation of transcription factors such as Hypoxia inducible factor -1 (HIF-1) (*Sakuda et al. 1992; Kourembanas et al. 1998; Berse et al. 1999; Chiarugi et al. 1999; Dachs et al. 2000*). In addition hypoxia has been shown to decrease and elastin and collagen synthesis in cultured aortic endothial cells, VSMCs and fibroblasts (*Stavenow et al. 1987; Durmowicz et al. 1991; Herrick et al. 1996; Steinbrech et al. 1999*). Furthermore, the collagen that is synthesised by hypoxic cells is

abnormal because oxygen is needed for the hydroxylation of praline (*Herrick et al. 1996*).

Hypoxia has been shown to increase elastase production in cultured macrophages (*Campbell et al. 1983*). The gene for MMP-9 contains consensus hypoxia sensitive AP-1 and NFκB sites in its promoter region and levels of MMP-9 and MMP-2 have been shown to be increased in mice exposed to hypoxia (*Himelstein et al. 1998; Zaidi et al. 2002*). Similarly, in pulmonary interstitial tissue from hypoxic exposed rabbits and human keratinocytes, MMP-9 expression and protein levels were increased (*O'Toole et al. 1997; Miserocchi et al. 2001*). However in one study using mouse fibroblasts, MMP-9 expression and activity were decreased after exposure to hypoxia (*Saed et al. 2000*). Other studies have demonstrated an increase in expression of MMP-2 in rat hepatocytes subjected to hypoxia (*Chen et al. 2000*). In one study using human trophoblastic and breast cancer cells, hypoxia had no effect on either MMP-2 or MMP-9 expression, but their activity was increased due to a decrease in the levels of TIMP-1(*Canning et al. 2001*) These findings suggest that hypoxia may shift in the balance between MMPs and their inhibitors favouring increased MMP activity.

The hypothesis as outlined in **Section 2.4.4** was that hypoxia within the aortic wall leads to increased elastolytic activity via either an XOR and/or oxidant stress pathway. Published data suggests that MMP-2 and MMP-9 produced by VSMC are the two elastolytic enzymes involved in the pathogenesis of AAAs. There are no previous studies assessing the influence of hypoxia on either the expression of MMP-2 or MMP-9 or elastolytic activity in human VSMC, which would obviously be relevant in the pathogenesis of AAA

5.2 Aims

The aims of this study were;

1. To determine if experimental hypoxia alters the expression or level of XOR in VSMC in culture
2. To determine if experimental hypoxia alters the;
    a. expression of MMP-2 and MMP-9 in VSMCs in culture
    b. level of MMP-2 and MMP-9 in conditioned media from VSMCs
    c. elastolytic activity of conditioned media from cultured human VSMCs

5.3 Materials and methods

5.3.1 Tissues preparation and VSMC culture

Tissue was collected as described in **Section 3.81** and VSMCs were cultured from aortic explants as described in **Section 3.8.2** (*Patel et al. 1996a*) and passaged as described in **Section 3.8.3**

5.3.2 To determine if Hypoxia alters XOR expression or XOR protein in cultured human VSMCs

5.3.2.1 Experimental protocol

Cells from passage 3 were seeded into 6 well plates at a density of 100,000 cells per well (**Section 3.8.4**). Cells were incubated in 10 % FCS/DMEM for 24-48 hours before experimentation. VSMCs were characterised by immunocytochemistry with a mouse monoclonal anti-α smooth muscle actin alkaline phosphatase conjugate antibody (**Section 3.8.6**). In addition cells were seeded onto LabTec slides for immunocytochemical analysis for XOR protein (**Section 3.8.7**)

Control (Time 0 (T_0)) total RNA, protein and unconditioned media (media blank) were retained at the beginning of the experiment. 0.5 ml of standard DMEM was added per well and plates were incubated in either 1% oxygen, balanced nitrogen/ CO_2 in a hypoxic chamber at 37° C (hypoxia group) or an atmosphere of 5% CO_2, 95% air at 37° C (normoxia group).

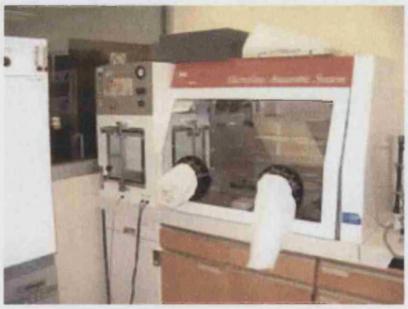

Figure 5-1 Hypoxic cabinet used for all experiments

5.3.2.2 Experiment end-points

At 2 hrs (T_2) and 6 hrs (T_6), total RNA and protein were isolated by adding 0.5 ml of RNA STAT 60 per well. RNA was isolated, quantified and reversed transcribed as described in **Section 3.9.1-3** and subjected to XOR and GAPDH RT PCR as described in **Section 3.9.4**. Expression of XOR mRNA was quantified as described in **Section 3.9.5.** Results are expressed as XOR/GAPDH ratio.

Total protein was isolated from RNA STAT 60 preparation as described in **Section 3.9.1.2** and quantified using the Bradford protein assay (**Section 3.4.2**). Samples were subjected to SDS-PAGE and Western blotting as described in **Section 3.4.3 and 3.4.4** At 24 hrs (T_{24}), 0.5 ml of protein lysing buffer (**Section 3.4.1**) was added per well and cell suspensions made by repetitive pippetting through a 23 G needle. Samples were centrifuged at 10,000 rpm for 10 minutes and supernatants retained. Total protein was quantified using the Bradford assay (**Section 3.4.2**). Levels of XOR protein were semi quantified using the immunoblot assay as described in **Section 3.5.1**
Conditioned media was collected at each time point and was used in the SAAANA elastolytic activity assay as described in **Section 3.11.1**.

5.3.2.3 Experimental number

No formal power calculation could be undertaken for this experiment as no previous studies have been reported. The experiment was carried out in triplicate for each experimental condition and repeated 4 times (N=4)

5.3.3 To determine if hypoxia alters MMP-2 or MMP-9 expression/levels or elastolytic activity in cultured human VSMCs

5.3.3.1 Experimental protocol

Only cells derived from aortic tissue from non-AAA patients, ie cadaveric organ donors were used for this experiment. Cells from passage 3 were seeded into T_{25} culture flasks at a density of 2.5 x 10^5 cells per flask or a 6 well plate at a density of 100,000 cells per well (**Section 3.8.4**). Cells were incubated in standard 10 % FCS/DMEM for 24-48 hours and then washed with PBS to remove any residual serum and then further cultured in serum-free DMEM containing BSA (0.1 mg/ml), Ascorbate (100uM), Insulin-Transferin-Selenium A supplement 100x (GibcoBRL Life Technologies), Penicillin (100 IU/ml), Streptomycin (100µg/ml) and L-Glutamine (0.2mM). VSMCs were characterised by immunocytochemistry with a mouse monoclonal anti-α smooth muscle actin alkaline phosphatase conjugate antibody (Sigma) (**Section 3.85**)

5.3.3.2 Experimental end-points

5.3.3.2.1 MMP-2/9 mRNA expression

Cells seeded in 6 well plates at a density of 100,000 cells per well were used for this experiment. Control total RNA was retained at the beginning of the experiment (Time 0 (T0)). 1 ml of either serum-free DMEM or standard 10% v/v FCS/DMEM was added per well and cells incubated in either 1% oxygen balanced nitrogen/ CO_2 in a hypoxic

chamber at 37°C (hypoxia group) or an atmosphere of 5% CO_2, 95% air at 37°C (normoxia control group).

At 2 hr (T_2) and 6 hrs (T_6), total RNA was isolated from cultured VSMCs cells by the addition of 0.5 ml of RNA STAT 60. RNA was isolated, quantified and reversed transcribed as described in **Section 3.9.1-3** and subjected to MMP-2, MMP-9 and GAPDH RT PCR as described in **Section 3.9.4**. Expression of MMP-2 and MMP-9 mRNA were quantified as described in **Section 3.9.5.** Results are expressed as product/GAPDH ratio.

5.3.3.2.2 MMP2/9 protein and elastolytic activity

Cells seeded in T_{25} culture flasks at a density of 2.5×10^5 cells per flask were used for this experiment. Unconditioned serum-free DMEM was retained at the beginning of the experiment (Time 0 (T_0)). 2 ml of serum-free DMEM were added per T_{25} flask and cells incubated in either 1% oxygen balanced nitrogen/ CO_2 in an hypoxic chamber at 37°C (hypoxia group) or an atmosphere of 5% CO_2, 95% air at 37°C (normoxia group) for either 24 hours (T_{24}) or 48 hours (T_{48}). Conditioned media was collected from each time point and used for MMP-2 and MMP-9 ELISAs **(Section 3.10)** as well as in SAAANA elastolytic activity assay **(Section 3.11.1)** and the MMP gelatinase activity assay **(Section 3.11.2)**.

5.3.3.3 Experimental number

No formal power calculation could be undertaken for this experiment as no previous studies have been reported. The experiment was carried out in triplicate for each experimental condition and repeated 4 times (N=4)

5.3.4 Data presentation and statistical analysis

5.3.4.1 Controls

Data were analysed at identical time points, ie hypoxia was compared to normoxia controls.

5.3.4.2 Data presentation and statistical analysis

Statistical analysis was performed using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Data were assessed for deviations from Gaussian distribution using the Kolmogorov-Smirnov test. Continuous variables were expressed as either means ± standard deviation (SD) or Standard error of the mean (SEM) for parametric data. Variables were analysed with students t-test. Statistical significance was considered for p values less than 0.05.

5.4 Results

5.4.1 Characterisation of vascular smooth muscle cells

The identification of VSMCs in culture was confirmed by the typical 'hill and valley' appearance of VSMCs in culture and immunopositivity for α actin (**Figures 5.2 and 5.3**).

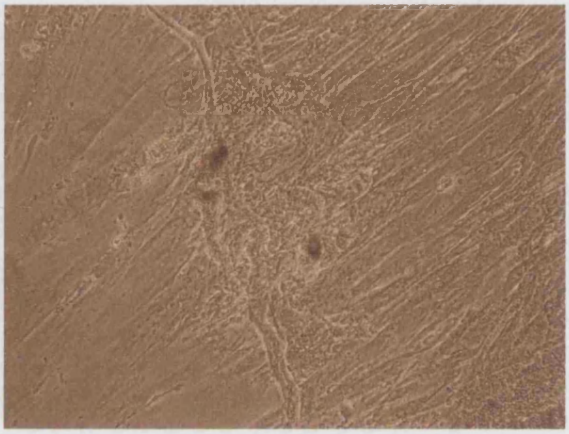

Figure 5-2 Phase contrast micrograph of vascular smooth muscle cells (VSMC) (Magnification x100)

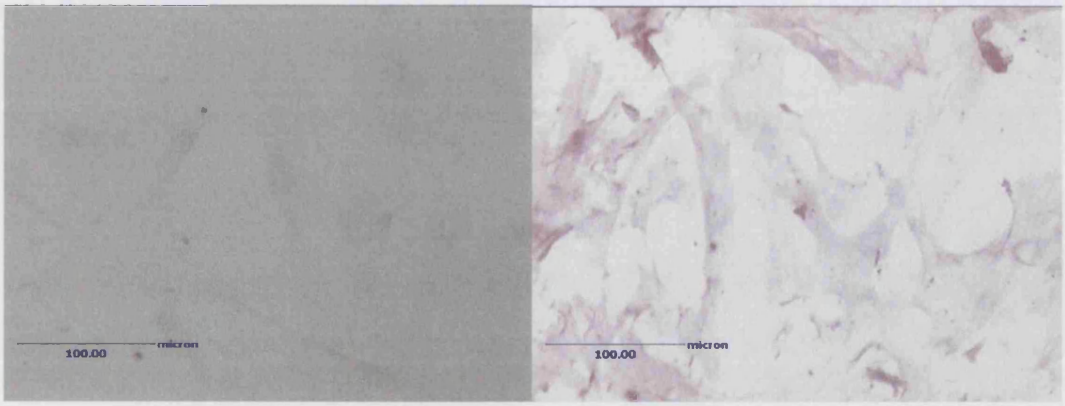

Figure 5-3 Cultured cells showing immunopositivity for α-actin

Image on left is negative control. The cells were incubated with diluted (1:25) mouse monoclonal anti-α smooth muscle actin alkaline phosphatase conjugated antibody (Sigma). Control slides were incubated with the blocking buffer alone and an irrelevant alkaline phosphatase conjugated antibody. The colour was developed with Sigma Fast Red™ and counterstained with Mayer's haematoxylin.

Vascular smooth muscle cells were confirmed as a source for xanthine oxidase by immunopositivity for XOR (Fig 5.4).

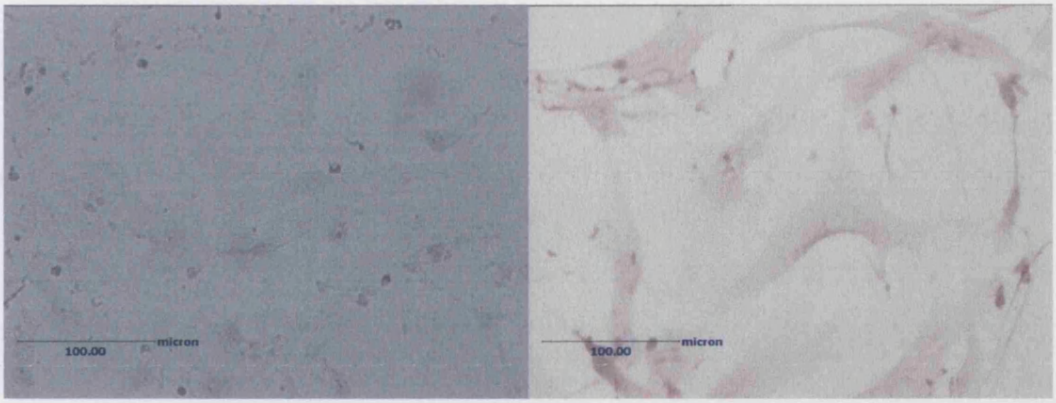

Figure 5-4 Cultured cells showing immunopositivity for XOR.

Image on left is negative control. Cells were incubated with diluted rabbit polyclonal anti-bovine XOR antibody (Chemicon, 1:100) and a biotinylated goat anti-rabbit IgG secondary antibody (Vector Labs, 1:100) followed by a 1:100 dilution of an alkaline phosphatase-conjugated avidin biotin complex (ABC) (Vectastain, Vector Labs). The colour was developed with Sigma Fast Red™ and counterstained with Mayer's haematoxylin

5.4.2 Effect of hypoxia on xanthine oxidase expression and protein levels

5.4.2.1 XOR mRNA expression

Mouse liver cDNA was utilized as a positive control for XOR PCR. Preliminary data confirmed that PCR for GAPDH and XOR worked and the PCR product for GAPDH was identified as a band corresponding to 576 base pairs and XOR as a product at 288 base pairs as predicted from the position of the primers on the sequence.

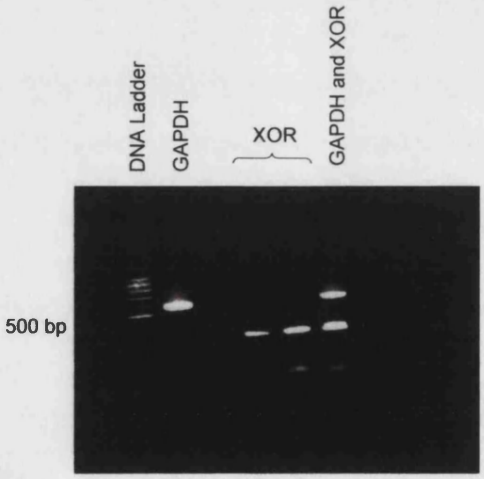

Figure 5-5 PCR for XO and GAPDH using mouse liver c DNA

The PCR product for GAPDH was visualised as a product at 576 base pairs,and the product for XOR was visualised as product at 288 base pairs.

All PCR for GAPDH and XOR were run on separate gels. Individual experiments were run on the same gel to minimise inter-gel variation. Results are expressed as XOR/GAPDH ratio.

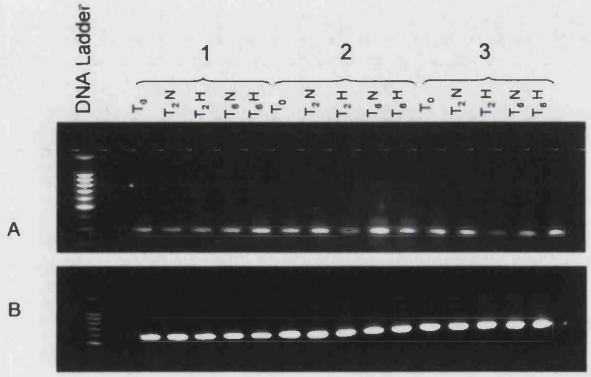

Figure 5-6 Representative PCR for XO (A) and GAPDH (B) for VSMCs

The experiment was carried out in triplicate for each experimental condition and repeated 4 times (N=4).

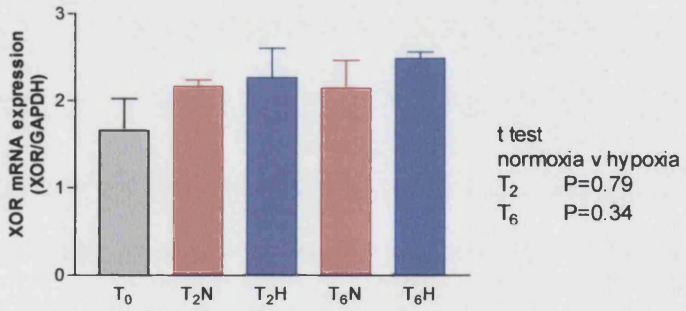

Figure 5-7 XOR mRNA expression of cultured VSMC exposed to normoxia and hypoxia for 6 hours.

Data are mean ± SEM of 4 experiments in triplicate. Data were analysed at identical time points, ie hypoxia was compared to normoxia controls at T_2 and T_6

The data from this experiment indicate no significant difference in XOR mRNA expression between VSMCs stimulated with 1% hypoxia or normoxia either at 2 or 6 hours. The data suggest that 1% hypoxia has no effect on XOR expression in cultured human VSMCs at 2 or 6 hours.

5.4.2.2 XOR protein expression

RNA STAT 60 protocol

No protein bands corresponding to XOR protein were visible in any experimental samples tested. Identical results were obtained when the membrane were probed with an anti XO HRP conjugated antibody (Data not shown). No further analysis of these samples could be undertaken.

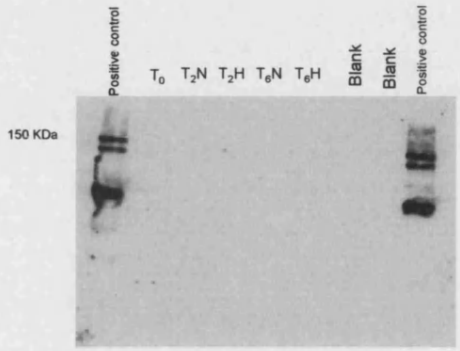

Figure 5-8 Western blot of STAT-60 protein preparations

Commercially available bovine XOR enzyme was used as a positive control. 15µg of total protein were loaded per lane. Membranes were probed with a rabbit polyclonal XO antibody (Chemicon, 1:500) and a secondary conjugated horseradish peroxidase swine-anti rabbit antibody (Dako, 1:1000 dilution). Characteristic XOR protein bands were only present in positive controls. Experiment samples contained no XOR protein bands.

T_{24} VSMC protein lysates

Levels of XOR protein in protein lysates from VSMCs exposed to either hypoxia or normoxia for 24 hours were semi quantified using the immunoblot assay as described in **Section 3.5.1.** Fifteen (15µg) micrograms of total protein were loaded per well. Biozyme bovine XOR enzyme used as positive control (15µg/well). Blots probed with rabbit anti-XOR (Chemicon 1:1000 dilution) and conjugated horseradish peroxidase swine-anti rabbit (Dako, 1:1000 dilution). Data are mean ± SEM of 4 experiments in triplicate. No statistical difference was observed between XOR protein levels at 24 hours in VSMCs either exposed to normoxia or hypoxia.

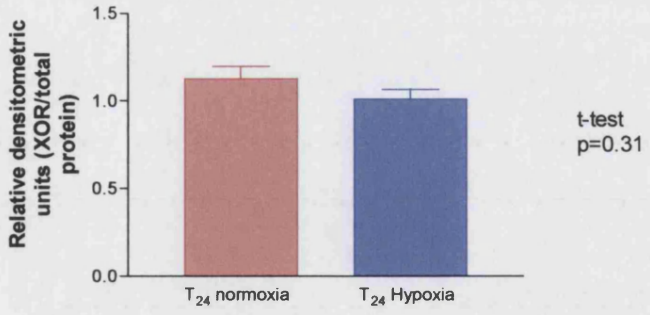

Figure 5-9 XOR protein levels (relative densitometry units) in VSMC stimulated with hypoxia

Data are mean ± SEM of 4 experiments in triplicate.

5.4.2.3 Elastolytic activity. - Succinyl trialanyl 4-nitroanilide (SAAANA) assay

The sensitivity of the assay determined from the standard curve using porcine pancreatic elastase in buffer was 20ng/ml.

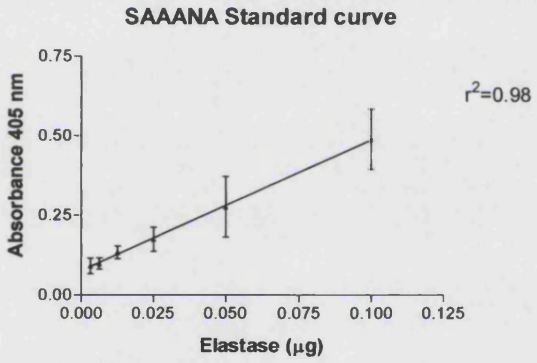

Figure 5-10 Standard curve for SAAANA assay

Porcine pancreatic elastase (PPE) diluted in unconditioned experimental media.

The absorbance value for unconditioned media (sample blank, i.e. no elastase) was greater than the absorbance value for experimental samples, indicating that samples contained no measurable elastolytic activity. There were no differences between Normoxia and hypoxia samples at either T_2 or T_6.

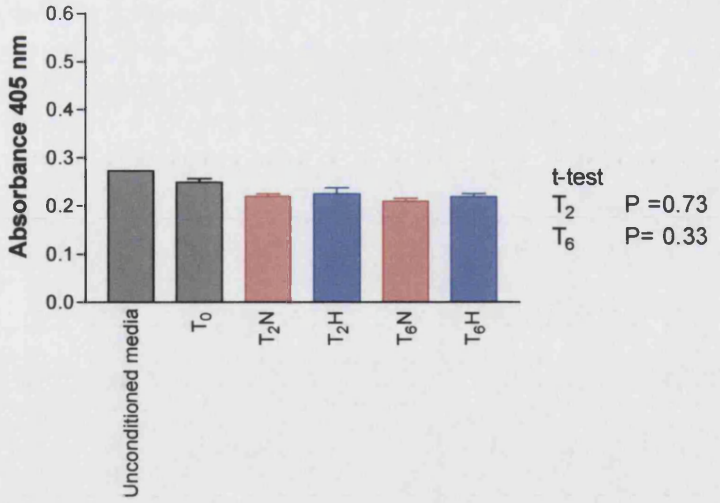

Figure 5-11 Absorbance values of conditioned media

Absorbance values for unconditioned media were similar as for experimental samples indicating no measurable elastolytic activity present. Data are mean ± SEM of 4 experiments in triplicate.

Possible reasons to explain as to why no elastolytic activity was demonstrated in VSMC cultures using this assay are;

1. The phenol red in the DMEM interfered with the absorbance
2. The Foetal calf serum (FCS) colour interfered with the assay
3. The presence of FCS had an anti-elastase activity thus inhibiting the assay
4. T_2 and T_6 were not long enough time points for elastase enzymes to be secreted into the media
5. Elastolytic enzymes maybe in the latent (non-active) form.

In respect to the first two suggestions, if the standard curve was repeated with the porcine pancreatic elastase (PPE) diluted in DMEM/ 10% FCS rather than buffer, a steeper standard line is obtained.

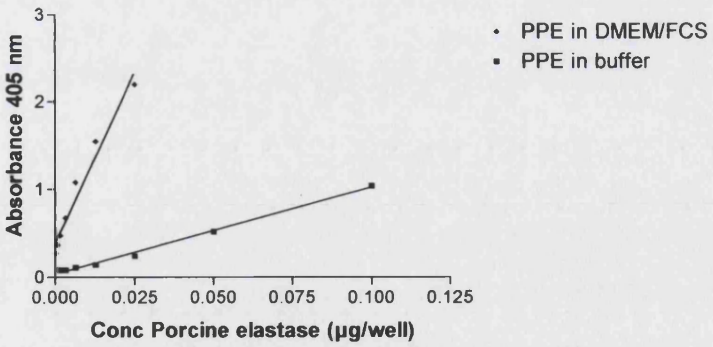

Figure 5-12 Standard curve for porcine pancreatic elastase diluted in either assay buffer or 10% FCS/DMEM

This observation would suggest that the colour of the DMEM/FCS interferes with the absorbance at 405 nm. This result suggests that DMEM/FCS does not inhibit the assay, because for each concentration of PPE, a higher absorbance is seen with DMEM/FCS rather than buffer alone

5.4.3 Effect of hypoxia on MMP-2 and MMP-9 expression, protein levels and elastolytic activity in cultured human VSMCs

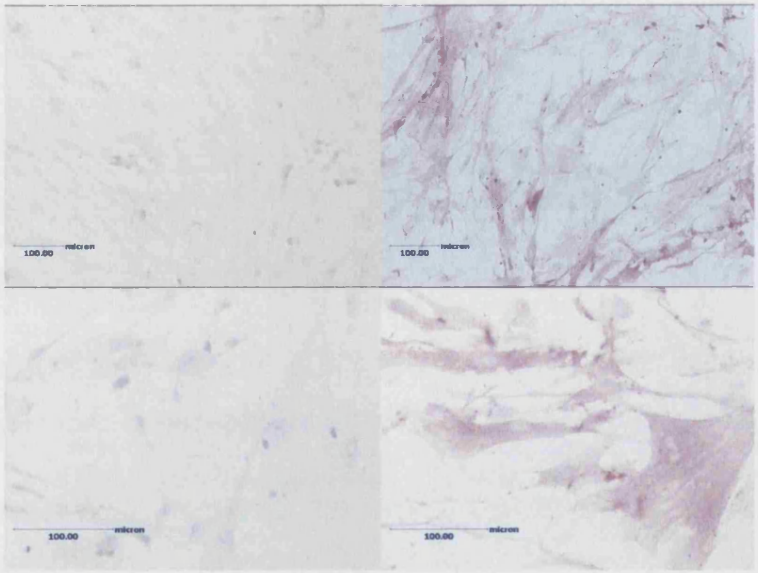

Figure 5-13 Cultured cells grown in serum free media showing immunopositivity for α-actin.

Image on left is negative control. The cells were incubated with diluted (1:25) mouse monoclonal anti-α smooth muscle actin alkaline phosphatase conjugated antibody (Sigma). Control slides were incubated with the blocking buffer alone and an irrelevant alkaline phosphatase conjugated antibody. The colour was developed with Sigma Fast Red™ and counterstained with Mayer's haematoxylin.

5.4.3.1 Hypoxia and MMP expression

5.4.3.1.1 MMP-2

A representative PCR for MMP-2 from VSMC in serum free media is shown below. The experiment was performed in triplicate and repeated on 3 occasions. Results are expressed as MMP-2/GAPDH ratio.

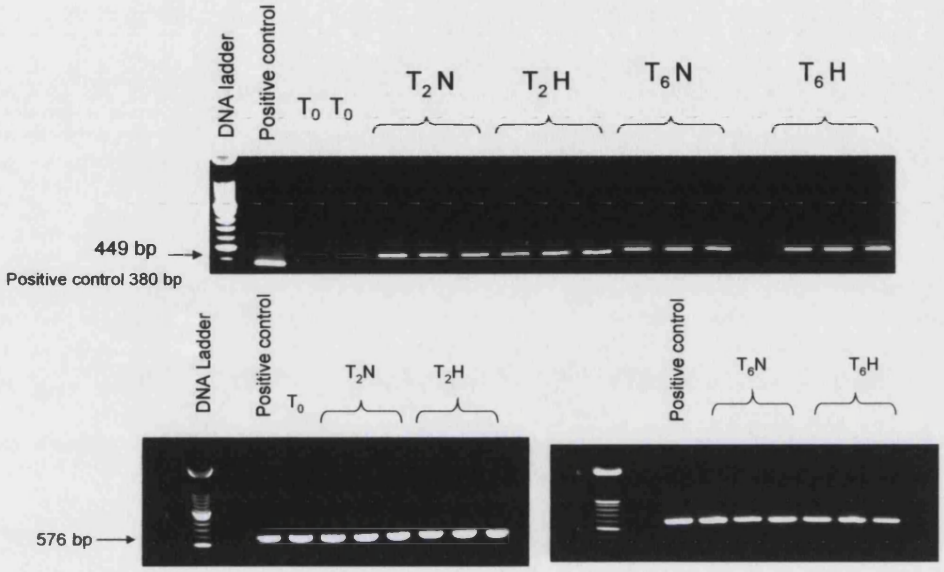

Figure 5-14 Representative MMP-2 PCR in serum free media.

The PCR product was visualised at 449 bp. Corresponding GAPDH PCR products are shown on the lower 2 gels.

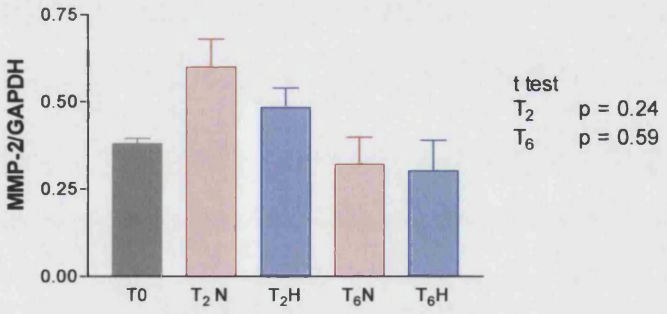

Figure 5-15 MMP-2 mRNA expression of cultured VSMC in serum free media exposed to normoxia and hypoxia for 6 hours

Data are mean ± SEM of 3 experiments in triplicate. Data were analysed at identical time points, ie hypoxia was compared to normoxia controls at T_2 and T_6.

The data from this experiment indicate that MMP-2 expression in cultured VSMCs is not influenced by hypoxic conditions. Similar results were obtained in parallel experiments performed with VSMCs in 10% FCS/DMEM exposed to normoxia or hypoxia for up to 6 hours.

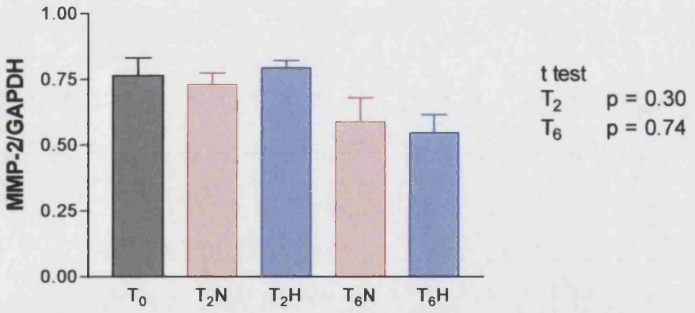

Figure 5-16 MMP-2 mRNA expression of cultured VSMC in 10% FCS/DMEM exposed to normoxia and hypoxia for 6 hours

Data are mean ± SEM of 3 experiments in triplicate. Data were analysed at identical time points, ie hypoxia was compared to normoxia controls at T_2 and T_6.

5.4.3.1.2 MMP-9

No MMP-9 PCR product was detected in any of the samples analysed.

The fact that the PCR for GAPDH revealed PCR products of the expected size, and the fact that the positive control for MMP-9 worked, all confirmed that mRNA, reverse transcription and PCR were adequate. There were no differences if the experiment was performed in serum free or serum containing media. The conclusion from this is that VSMC in culture did not express MMP-9.

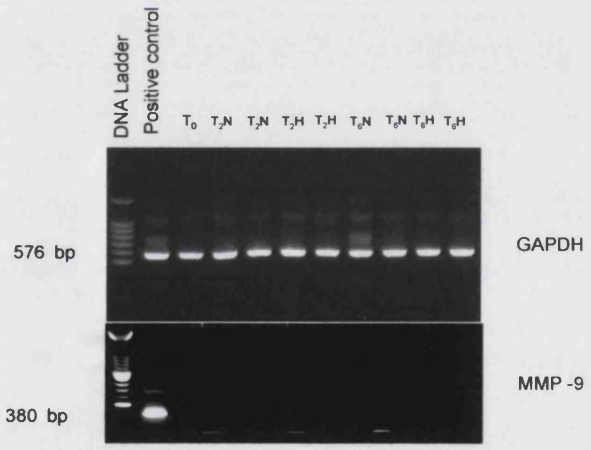

Figure 5-17 Representative MMP-9 PCR from VSMCs in serum free media

The PCR product for GAPDH was visualised at 576 bp. No experimental MMP-9 PCR product was visualised.

5.4.3.2 Hypoxia and MMP-2/9 protein levels and elastolytic activity

5.4.3.2.1 MMP-2 ELISA and MMP-9 ELISA

Conditioned media from experiment 2 were used in the MMP-2 and MMP-9 ELISAs. The manufacturers' protocol was followed.

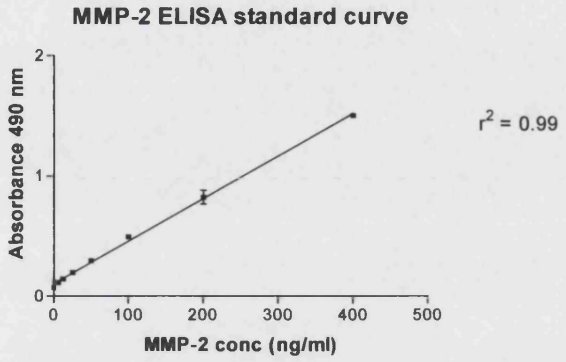

Figure 5-18 MMP-2 ELISA standard curve

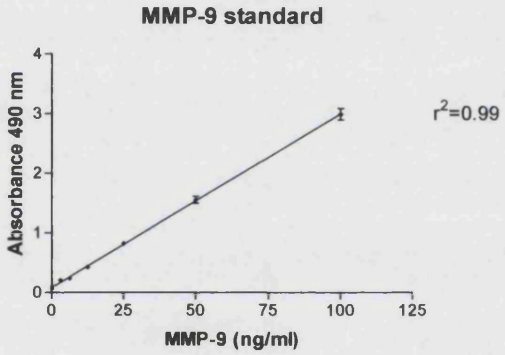

Figure 5-19 MMP-9 ELISA standard curve

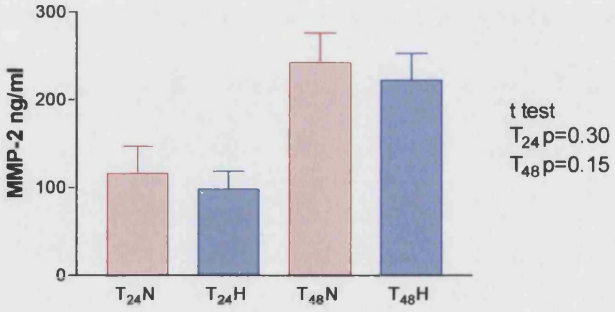

Figure 5-20 Results of MMP-2 ELISA

Data are mean ± SEM of 4 experiments in triplicate. Data were analysed at identical time points, ie hypoxia was compared to normoxia controls at T_{24} and T_{48}.

VSMCs in culture both showed increased MMP-2 protein levels over 24-48 hours but this increase was the same for hypoxia and normoxia. No difference was detected in MMP-2 protein levels at either 24 or 48 hours incubation in hypoxia compared to normoxia.

No MMP-9 was detected in any samples analysed. All absorbance values were lower than the sensitivity of the assay, i.e. 3.1ng/ml of MMP-9

5.4.3.2.2 Succinyl trialanyl 4-nitroanilide (SAAANA) assay

Conditioned media samples from experiment **5.3.3.2.2** were used in this assay. Due to the problems mentioned in **Section 5.4.2.3** with this assay, experiments were conducted in DMEM without phenol red and in the absence of foetal calf serum. Assay was carried out as described by Patel at pH 7.2 (*Patel et al. 1996a*).

A typical standard curve obtained from porcine pancreatic elastase (PPE) diluted in unconditioned media is shown in **Fig 5.21**

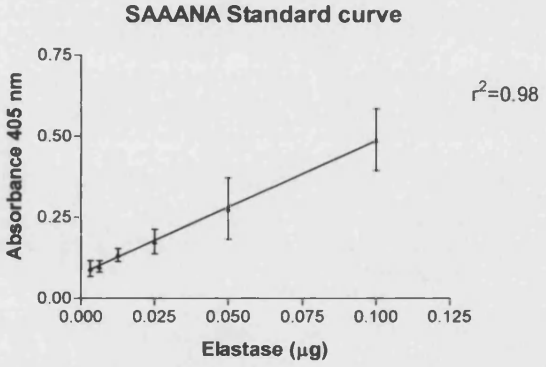

Figure 5-21 Standard curve for SAAANA assay.
Porcine pancreatic elastase (PPE) diluted in unconditioned experimental media was used

Despite the generation of the standard curve as demonstrated above, a problem was found with the use of this assay when experimental samples were assayed.

Multiple wells (> 75%) developed a non-specific 'cloudiness'. The 405 nm readings were suggestive of a positive reaction, however, these assay wells also gave high reading with a 595 nm non-specific filter, confirming that the data were false positive values and not valid.

Initially I believed that the cloudiness was due to infection within the experimental media samples, however despite filtering all reagents used in the assay the cloudiness remained and was noted to be due to a precipitate in the bottom of each well.

In investigating this further I determined whether this was a pH related effect. According to Patel's description the best pH for this assay is 7.2, and that was the buffer pH that I originally used (*Patel et al. 1996a*). In order to test this variable, a range of TrisHCL buffers (7.2-8.4) were used. In addition the presence/absence of media and the presence/absence of $CaCl_2$ were investigated. Porcine pancreatic elastase was used as a positive control (10µg/well). 200µl of buffer were added to 100µl media/H_2O/elastase and 10µl of SAAANA was added. Experiments were incubated for 4 hours at 37°C.

	Media		H2O		Elastase/Media		Elastase/H2O	
Buffer								
	405 nm	595 nm	405 nm	595 nm	405 nm	595 nm	405 nm	595 nm
7.2	0.073	0.038	0.061	0.035	1.825	0.049	1.795	0.044
7.2 $CaCl_2$	0.076	0.04	0.056	0.033	1.85	0.05	1.82	0.046
7.4	0.075	0.04	0.058	0.035	2.026	0.05	1.8	0.045
7.4 $CaCl_2$	0.076	0.041	0.058	0.034	1.861	0.05	1.786	0.045
7.6	0.073	0.038	0.059	0.035	1.888	0.048	1.803	0.046
7.6 $CaCl_2$	0.079	0.04	0.058	0.034	1.822	0.05	1.843	0.044
7.8	0.077	0.041	0.056	0.032	1.813	0.048	1.803	0.044
7.8 $CaCl_2$	0.142	0.072	0.06	0.034	1.9	0.074	1.826	0.045
8.0	0.076	0.04	0.059	0.034	1.832	0.048	1.812	0.045
8.0 $CaCl_2$	0.156	0.076	0.059	0.035	1.898	0.077	1.856	0.047
8.2	0.076	0.04	0.058	0.034	1.845	0.047	1.79	0.044
8.2 $CaCl_2$	0.275	0.13	0.058	0.034	1.979	0.101	1.837	0.046
8.4	0.08	0.043	0.061	0.036	1.837	0.046	1.783	0.043
8.4 $CaCl_2$	0.258	0.122	0.064	0.036	1.872	0.106	1.792	0.044

Table 5-1 Effects of TrisHCL buffer (7.2-8.4) on SAAANA assay

The results of this experiment show that the combination of pH > 7.8, media and calcium chloride result in non-specific 'cloudiness' even in the absence of any elastase. In a second experiment, longer incubations were observed to determine if time influenced the development of the precipitate. Porcine pancreatic elastase was used as a positive control (0.1µg/well). 200µl of buffer were added to 100µl sample (media +/- elastase) and 10µl of SAAANA was added. Experiments were incubated for 48 hours at 37°C.

	Media			Elastase/Media	
T_0					
	405 nm	595 nm		405 nm	595 nm
7.2 $CaCl_2$	0.072	0.037		0.083	0.038
7.4 $CaCl_2$	0.073	0.038		0.084	0.038
7.6 $CaCl_2$	0.077	0.041		0.083	0.038
7.8 $CaCl_2$	0.083	0.042		0.087	0.04
8.0 $CaCl_2$	0.085	0.042		0.088	0.039
8.2 $CaCl_2$	0.218	0.101		0.23	0.099
8.4 $CaCl_2$	0.197	0.093		0.212	0.093
T_{24}					
	405 nm	595 nm		405 nm	595 nm
7.2 $CaCl_2$	0.089	0.04		2.297	0.064
7.4 $CaCl_2$	0.099	0.045		2.311	0.05
7.6 $CaCl_2$	0.137	0.08		2.261	0.08
7.8 $CaCl_2$	0.186	0.102		2.29	0.098
8.0 $CaCl_2$	0.197	0.101		2.327	0.099
8.2 $CaCl_2$	0.278	0.129		2.376	0.133
8.4 $CaCl_2$	0.273	0.132		2.394	0.152
T_{48}					
	405 nm	595 nm		405 nm	595 nm
7.2 $CaCl_2$	0.111	0.08		2.302	0.069
7.4 $CaCl_2$	0.126	0.09		2.301	0.09
7.6 $CaCl_2$	0.165	0.095		2.296	0.093
7.8 $CaCl_2$	0.165	0.099		2.222	0.099
8.0 $CaCl_2$	0.178	0.102		2.27	0.12
8.2 $CaCl_2$	0.22	0.128		2.278	0.132
8.4 $CaCl_2$	0.201	0.129		2.396	0.149

Table 5-2 Effect of time on incubations for SAAANA assay

The data demonstrate that the non-specific 'cloudiness' developed in lower pH buffers with longer incubation periods.

From these observations we believe that the presence of calcium chloride, which is required for MMP activation, causes something in the media (probably the ascorbic acid) to precipitate as the pH rises with prolonged incubation times.

In an effort to overcome these non-specific 'colour' changes, experimental samples were 'blanked' by subtracting the 595 nm reading from 405 nm reading as well as subtracting the unconditioned media/buffer/SAAANA reading. When this was performed the results from this assay would suggest that no elastolytic activity could be demonstrated in any samples measured using this technique.

5.4.3.2.3 Gelatinase activity assay

Due to the problems with the SAAANA assay this commercially available assay was used to quantify the elastolytic activity of conditioned media from cultured VSMCs exposed to either hypoxic or normoxic experimental conditions.

The results are expressed as a percentage activity of the positive control provided ((Positive control OD/Sample OD) x 100 = percent activity) and as a quantitative result by comparison with the activity of purified MMP-2 (Chemicon CC071) activated by *p*-Aminophenylmercuric Acetate (APMA) using the protocol described (Chemicon ECM 701).

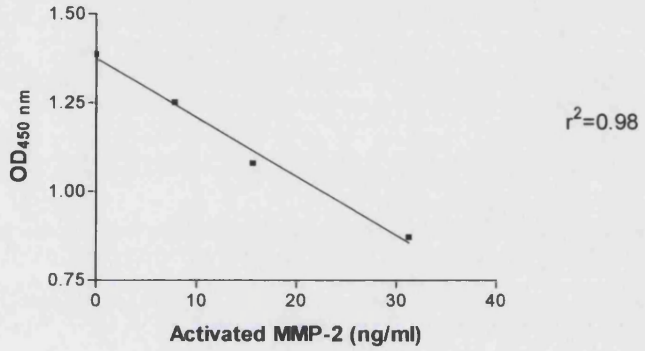

Figure 5-22 Standard curve for APMA activated MMP-2 in the gelatinase activity assay (Chemicon)

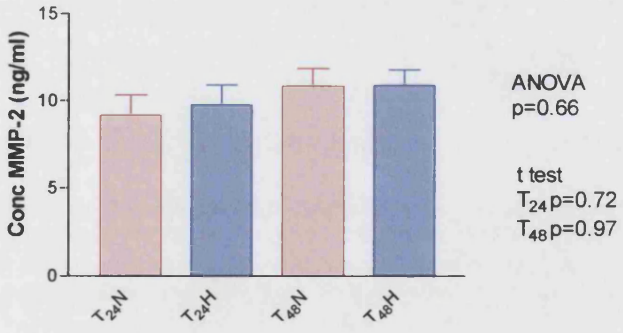

Figure 5-23 Quantitative gelatinase activity of conditioned media from VSMCs exposed to either hypoxia or normoxia for up to 48 hours

Data are mean ± SEM of 4 experiments in triplicate.

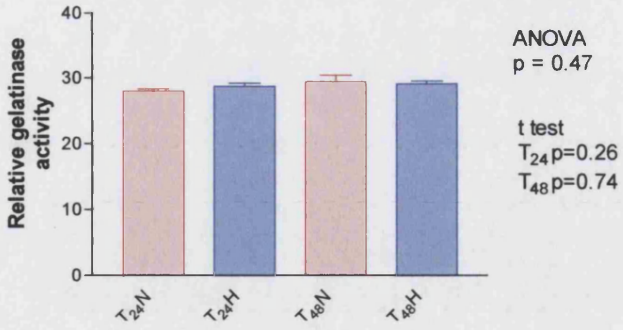

Figure 5-24 Relative gelatinase activity of conditioned media from VSMCs exposed to either hypoxia or normoxia for up to 48 hours

Data are mean ± SEM of 4 experiments in triplicate.

Using the Chemicon gelatinase activity assay, no difference in gelatinase activity could be detected between VSMCs exposed to either hypoxia or normoxia for up to 48 hours.

5.5 Discussion

Matrix metalloproteinases, in particular MMP-2 and MMP-9 are believed to play a prominent role in the initiation and development of ECM changes that lead to aneurysmal dilatation (Chapter 2).

MMP-2 derived from VSMCs is believed to be the dominant elastolytic enzyme in the wall of small early aneurysms, with MMP-9 becoming more prominent as the inflammatory infiltrate increased in density (*Freestone et al. 1995; Thompson et al. 1995; McMillan et al. 1997; Petersen et al. 2000; Petersen et al. 2002*). The reasons for the elevated MMP-2 gene transcription in aortic VSMCs derived from patients with AAA are unclear. No mutational abnormalities in the MMP-2 gene itself have been reported, and the level of expression of MMP-2 is not readily modulated, because it is constitutively expressed and exhibits the characteristics of a housekeeping gene (*Huhtala et al. 1990; Galis et al. 1994*). The MMP-2 promoter lacks a TATA box and AP-1 transcriptional binding motif common to other inducible MMPs, however Davis demonstrated that MMP-2 production was most prominent in areas of inflammation in AAAs and suggested paracrine modulation of the MMP-2 gene (*Gaire et al. 1994; Davis et al. 1998*). MMP-2 has been shown to be up-regulated in rat glomerular mesangial cells due to a cis-acting enhancer element in the rat MMP-2 gene which binds the transcription factors AP-2 and YB-1 (*Harendza et al. 1995; Mertens et al. 1998*) and in response to exogenous cytokinetic stimuli (*Marti et al. 1994*). This has never been demonstrated in human MMP-2. An alternative explanation may be that there is polymorphism in the MMP-2 gene or promoter region and indeed Price has recently identified 6 polymorphisms in the human MMP-2 promoter region, one of which, a C-T transition at -1306 was associated with differential promoter activity (*Price et al. 2001*). The relationship of MMP-2 polymorphism with AAA remains to be clarified and is the subject of ongoing investigations (*Goodall et al. 2001*).

In contrast to MMP-2, the gene for MMP-9 contains TATA and CAAT boxes, AP-1, NFκB-binding sites and PEA-3 elements in the 5'-flanking region of the gene (*Huhtala et al. 1991; Ye 2000*). MMP-9 is principally produced by inflammatory macrophages, but AAA VSMCs do express MMP-9 in culture and expression is increased in response to inflammatory cytokines such as IL-1β (*Evans et al. 1991; Galis et al. 1994*). Interestingly polymorphisms of MMP-9 have been studied in AAA patients, but no association has been detected (*Yoon et al. 1999; Ye 2000*).

Hypoxia has been shown to increase elastase production in cultured macrophages and levels of MMP-9 and MMP-2 have been shown to be increased in mice exposed to

hypoxia (*Campbell et al. 1983; Himelstein et al. 1998; Zaidi et al. 2002*). In addition, pulmonary interstitial tissue from hypoxic exposed rabbits had raised MMP-9 expression and protein levels (*Miserocchi et al. 2001*) and MMP-2 expression has been shown to be increased in rat hepatocytes subjected to hypoxia (*Chen et al. 2000*). In one study using human trophoblastic and breast cancer cells, hypoxia had no effect on either MMP-2 or MMP-9 expression, but their activity was increased due to a decrease in the levels of TIMP-1 (*Canning et al. 2001*). These findings suggest that hypoxia may shift in the balance between MMPs and their inhibitors favouring increased MMP activity. Based on this previous evidence and the potential link between these factors especially hypoxia, MMP expression and elastolytic activity, I decided to simulate hypoxia in cultured human VSMCs to determine if this stimulus could account for elevated MMPs and elastolytic activity. The principle aim of this study was to investigate a possible relationship between hypoxia, MMP expression and elastolytic activity in VSMCs in culture.

Cell culture has the advantage of allowing the study of isolated cell lines under controlled conditions (*Carrell et al. 1999*). Vascular smooth muscle cells cultured from aneurysms have been shown to be a source of the elevated production of both MMP-2 and MMP-9 and were thus chosen as the study source (*Evans et al. 1991; Galis et al. 1994; Keen et al. 1994; McMillan et al. 1995; Crowther et al. 1996; Patel et al. 1996b; Crowther et al. 2000a*). The potential disadvantage of isolated cell culture techniques is that the interaction and possible paracrine influence of other cell lines is eliminated and thus the model may be criticised as not reflecting *in vivo* conditions, which is why other investigators have developed techniques of aortic tissue ex-vivo explants (*Wills et al. 1996*).

A major assumption in this study is that hypoxia exists in the wall of aortic aneurysms. Whilst data exist to support this assumption in experimental animal models, particularly with atherosclerosis, the data for human vessel hypoxia is limited, probably reflecting the difficulty in measuring this parameter. Vorp using an order-of-magnitude analysis and computational analysis calculated that the aortic wall in aneurysms is hypoxic (*Vorp et al. 1996; Vorp et al. 1998*). In a further study, reported since the completion of our study, Vorp demonstrated that the AAA wall adjacent to a thick layer of intraluminal thrombus (ILT) have localised areas of hypoxia. In this study patients with AAA were placed in one of two groups: (1) those with an ILT of 4-mm or greater thickness on the anterior surface or (2) those with little (< 4 mm) or no ILT at this site. During surgical resection but before aortic cross-clamping, a needle-type polarographic partial pressure

of oxygen (pO_2) electrode was inserted into the wall of the exposed aorta and the pO_2 was measured. The probe was advanced, and measurements were made midway through the thrombus and in the lumen. Mural and mid-ILT pO_2 measurements were normalized by the intraluminal pO_2 measurement to account for patient variability. In addition, AAA wall specimens were obtained and assessed for degree of inflammation, neovascularisation and tensile strength. Western blotting and immunohistochemistry for qualitative evaluation of expression of the cellular hypoxia marker oxygen-regulated protein was also performed. The pO_2 measured within the AAA wall in group I (n = 4) and group II (n = 7) patients was 18% +/- 9% luminal value versus 60% +/- 6% (mean +/- SEM; P <0.01). The normalized pO_2 within the ILT of group I patients was 39% +/- 10% (P =0.08 with respect to the group I wall value). Group I tissue specimens showed greater inflammation and degree of neovascularisation compared to group 2 and non-aneurysmal tissue. Tensile strength was significantly less for group 1 specimens. Western blotting and immunohistochemistry suggested that oxygen-regulated protein is more abundantly expressed in group I versus group II specimens. The conclusion from this study was that localized hypoxia occurs in the AAA wall in regions of thicker ILT. This localised hypoxia is associated with increased localized mural neovascularisation and inflammation, as well as regional wall weakening (*Vorp et al. 2001*).

Whilst this study demonstrates hypoxia in the wall of human aortic aneurysms it is worth pointing out that the numbers in the two groups are small. Points arising from this study that are worth highlighting are;

1. The question arises as to whether hypoxia is a primary etiological event or secondary consequence. Hypoxia was seen in both groups, but was most prominent in AAAs with large amounts of thrombus. Is it hypoxia that leads to AAA formation or AAA formation and ILT that leads to the confounding factor of hypoxia? The question as to whether hypoxia exists in non-aneurysmal vessels or in the early stage of aneurysm development remains unanswered.
2. Is the degree of hypoxia sufficient to alter cellular function?
3. The results from this study would suggest that hypoxia is associated with inflammation and neovascularisation, and it may be down this pathological pathway that hypoxia has a potential role in the development of AAAs. This theme was not the subject of this study but is obviously a potential channel for future research.

The data from this study demonstrates that:

1. VSMCs as a source of XOR in vascular tissue
2. The results from XOR RT PCR and XOR immunoblotting indicate that hypoxia had no effect on the expression or protein level of XOR.

Any potential association between hypoxia and elastolytic activity could theoretically be due to the function of this enzyme in conditions of hypoxia. It was decided not to measure XOR function in VSMCs at this stage because of the large number of cell numbers required to measure activity in cell cultures. It was decided that such measurements would only be required if there was a positive result in the hypoxia-elastolytic activity study.

An important observation from this first experiment was the difficulty with the SAAANA assay. The difficulties encountered with this assay and the possible causes for this are discussed in **Section 5.4.2.3 and 5.4.3.2.2.** A major concern from the first experiment was the possibility that either the foetal calf serum or phenol red within the media was interfering with the SAAANA assay. In order to overcome this it was decided to perform future experiments in serum free media without the presence of phenol red. As a consequence of using serum free media, all cells are synchronised at the same stage of the growth cycle (G_0). In addition to this, cells were seeded at a known and equal density at the beginning of each experiment. Cells were seeded at a density as described in previous VSMC experiments (*Patel et al. 1996b; Crowther et al. 2000a*). The effect of these two procedures was that both cell density and cell cycle stage were eliminated as variables that may account for any observed difference that may be found.

The results from our study on MMP2, MMP-9 expression and elastolytic activity in cultured VSMCs were:

1. VSMCs derived from aortic tissue only express MMP-2 but not MMP-9 in culture. In this particular experiment all VSMCs were derived from non-AAA control patients, ie normal cadaveric organ donors. Previous reports of cultured VSMCs expressing MMP-9 have been in VSMCs derived from AAA patients . VSMCs are known to express MMP-9 in response to cytokinetic influences and it may be this reason why VSMCs derived from AAA patients express MMP-9 . In the latter study MMP-9 was only detected in human VSMCs from saphenous vein if they were stimulated with interleukin-1α and either platelet derived growth factor (PDGF) or basic fibroblast growth factor (b FGF).
2. Hypoxia is unlikely to be a stimulus for MMP-9 expression in VSMCs.

3. Hypoxia did not influence the expression of MMP-2, or increase the protein levels of MMP-2 in VSMCs under the experimental conditions described.
4. Elastolytic activity in conditioned media from VSMCs can not be measured using the SAAANA elastolytic assay
5. Hypoxia did not lead to increased elastolytic activity as assessed by the Chemicon gelatinase activity assay.

The results from this study suggest that hypoxia does not influence the expression or activity of elastolytic MMPs. As stated earlier a major assumption in this study was that arterial wall hypoxia existed *in vivo*. A second point that may be criticized is that only one degree of hypoxia was used in the experiment and this may not actually be physiologically or pathologically relevant. It could be argued that perhaps a higher oxygen concentration or indeed a range of oxygen concentrations be studied before any firm conclusions can be drawn from this study. At the beginning of this study there were no published data on intramural oxygen tensions/pressures. I decided to choose our particular oxygen concentration because I believed that if there was an effect was to be seen with hypoxia and MMP expression/activity then it was most likely to be seen with severe oxygen tension, i.e. an all or nothing effect. I believe that as no effect was seen on either MMP-2 or MMP-9 expression or activity with such an extreme oxygen concentration, it is unlikely to be found with higher oxygen concentrations, however I do appreciate that I have not presented data to support this statement.

The hypothesis as discussed in chapter 3 was that hypoxia could lead to increased XOR activity which could then lead to increased elastolytic activity. A second assumption in this experimental model was that the confirmed presence of XOR expression in VSMCs implied functioning XOR enzyme. XOR function in hypoxic VSMCs was not measured during these experiments. The fact that the result of hypoxia on elastolytic activity was negative made the measurement of the influence of hypoxia on XOR activity in VSMCs unnecessary, as the link between hypoxia and increased elastolytic activity in VSMCs had not been proved.

The data suggest that the SAAANA assay described by Bieth and modified by Patel is not useful in the assessment of elastolytic activity (*Bieth et al. 1974; Patel et al. 1996b*). No elastolytic activity was detected in samples analysed, which either suggests that there was no MMP present in the samples, or the sensitivity of the test was too low. Another possibility is that the MMPs within the media samples were in the pro-enzyme form (latent form), which require activation. The finding that the MMP-2 ELISA confirmed the presence of MMP-2 enzyme and the finding that the Chemicon gelatinase

activity assay showed only low levels of endogenous MMP activity would suggest that the majority of MMP is in the latent form and the sensitivity of this assay is insufficient. Traditionally MMP expression and activity has been measured using a combination of semi-quantitative Western blotting and substrate gel enzymography. In addition to the measurement of MMPs, TIMPs analysis is required as net elastolytic activity will be a result of the balance between MMPs and their natural inhibitors. It is also worth pointing out that for MMPs to have elastolytic activity then they need to be in the active form. The disadvantage of most ELISA assays is that they are unable to distinguish between active MMPs and inactive zymogens. Substrate gel enzymography is sensitive and will detect in gel elastin digestion following gel electrophoresis. However, this process of elastase detection is not conducive to high capacity screening because it is limited by the number of wells that are available per gel, and it is time-consuming because it requires gel electrophoresis and protracted gel washing and in-gel digestion periods. In addition at best substrate gel enzymography is semi-quantitative. The relationship between the amount of enzyme and band intensity is not always linear and is a recognised problem with this technique (*Baragi et al. 2000*).

The MMP ELISA kits used in this study only detect pro-MMPs. The MMP-2 ELISA kit recognises both free pro-MMP-2 and pro-MMP-2 complexed with TIMP-2. It does not recognise active MMP-2. Likewise the MMP-9 kit recognises free pro-MMP-9, intermediate 83 KDa MMP-9 and MMP-9 complexed with TIMP-1. The assay does not recognise active MMP-9 (67 KDa) (Chemicon literature ECM 492/494)(*Okada et al. 1992; Fujimoto et al. 1993; Fujimoto et al. 1994*).

The Chemicon MMP Gelatinase Activity Assay Kit is ideal for measurement of net gelatinase activity. It only measures active MMPs, but is influenced by the amount of TIMPs present. Activity levels represent the net elastolytic activity, the balance between MMPs and their natural inhibitors. Note that unlike substrate gel zymography, the MMP gelatinase activity assay measures MMP activity in solution. The activity observed in natural solutions is often quite different from the apparent activity observed on zymographs, where the MMP enzymes are physically separated from their natural inhibitors. Analytical sensitivity is less than 5ng/ml of MMP, which is comparable to the analytical sensitivity of zymography.

If the MMP gelatinase assay is used in combination with the MMP ELISA assays then they provide quantitative data for pro-MMPs (inactive), active MMP and the net elastolytic activity and thus they negate the need for Western blotting and substrate gel zymography.

In conclusion, the results demonstrate that VSMCs express XOR and MMP-2 but not MMP-9 in culture. Hypoxia has no effect on XOR, MMP-2 or MMP-9 expression or protein levels in cultured VSMCs. In addition there is no evidence that hypoxia leads to increased production of elastolytic MMPs or increased elastolytic activity in VSMCs. These data would suggest that if hypoxia does have a role in the pathogenesis of aneurysm formation then it is not by directly increasing MMP expression and elastolytic activity in VSMCs.

Chapter 6 Oxidant stress and aortic aneurysms

6.1 Introduction

Oxidant stress is thought to be involved in atherosclerosis and particular plaque remodelling and is now beginning to be considered in AAA formation (*Zalba et al. 2000; Miller et al. 2002*). Reactive oxygen species have been reported to induce apoptosis of VSMCs which is a well described feature of AAA tissue (*Holmes et al. 1996; Johnson et al. 1996; Lopez-Candales et al. 1997; Henderson et al. 1999*). In addition the genes for MMP-1, MMP-3 and MMP-9 and the AAA associated cytokines including TNFα, IL-1, IL-6 all have AP-1 and NFκB binding elements in their promoter regions and thus are oxidant sensitive (*Huhtala et al. 1991; Sato et al. 1993; Vincenti et al. 1998; Allen et al. 2000; Griendling et al. 2000; Bond et al. 2001*). Indeed the expression of MMP-1 in human dermal fibroblast cultures (*Brenneisen et al. 1997*) and MMP-2 in cultured bovine aortic endothelial cells (*Inoue et al. 2001*) and MMP-9 in bovine aortic endothelial cells (*Uemura et al. 2001*) have all been shown to be modulated by oxidant stress. In addition, Galis showed that N-acetyl-L-cysteine, an anti-oxidant, decreased MMP-9 expression in foam cells from hypercholesterolaemic rabbits (*Galis et al. 1998*).

Previous studies have demonstrated that reactive oxygen and nitrogen species, including peroxynitrite activate the pro-enzyme forms of both MMP-2 and MMP-9 (*Rajagopalan et al. 1996; Okamoto et al. 1997; Galis et al. 1998; Maeda et al. 1998b; Buhimschi et al. 2000; Siwik et al. 2001; Uemura et al. 2001*). Interestingly the glutathione precursor N-acetylcysteine (an anti-oxidant) dramatically inhibited MMP-9 activity (*Buhimschi et al. 2000*). In addition to its effects on MMPs, peroxynitrite has been shown to inactivate TIMPs and alpha1-proteinase inhibitor, a major proteinase (neutrophil elastase) inhibitor in human plasma the net effect being accelerated tissue degradation (*Frears et al. 1996; Maeda et al. 1998b*)

Levels of ascorbic acid and antioxidant enzyme activities have been shown to be reduced in AAA tissue compared to non-aneurysmal tissue (*Dubick et al. 1999*). Furthermore, plasma levels of vitamin E, another anti-oxidant have been shown to be reduced in patients with AAA compared to patients with coronary artery disease without aneurysms (*Sakalihasan et al. 1996b*). These intriguing observations would support the hypothesis that oxidant stress is potentially increased in AAA tissue.

The hypothesis as outlined in **Section 2.4.4** was that increased oxidant stress within the aortic wall leads to increased elastolytic activity. There are limited studies investigating

oxidant stress and the potential role of reactive oxygen and nitrogen species in AAA formation other than those outlined above.

6.2 Aims

The aims of this study were to

1. To search for evidence of oxidant stress in AAA tissue.
2. To determine if oxidant stress activates MMPs in an *in vitro* model.

6.3 Materials and Methods

6.3.1 To demonstrate evidence of oxidant stress in aortic tissue

The demographics of the two groups of patients used in this study are given in **Table 6.1.** The samples were a sub-group from the previous used specimens (**Section 4.4.1**). No difference in gender distribution was noted but a statistically significant difference was seen with regard to age (Students t test, $p< 0.001$).

	AAA (n=18)	Control (n=13)	P
Age, y	73 (range 57-89)	44 (range 17-69)	P< 0.001
Gender, % male	82	62	P = 0.11

Table 6-1 Demographic data of patients

6.3.1.1 Peroxynitrite

Superoxide reacts with nitric oxide to form peroxynitrite. Peroxynitrite will cause diverse chemical reactions in biological system including nitration of tyrosine residues of proteins and the formation of free and protein 3-nitrotyrosine derivatives has been used as a probe for peroxynitrite formation (*Ischiropoulos 1998; Tarpey et al. 2001; Miller et al. 2002*).

6.3.1.1.1 Immunohistochemical detection of 3 nitrotyrosine (3-NT) in human aortic wall sections

Aortic tissue was collected and prepared as described in **Sections 3.2 and 3.3**. Immunohistochemical staining for nitrotyrosine was performed using the Vectastain ABC-AP kit (Vector Laboratories). An anti-nitrotyrosine antibody (Upstate Biotechnology Inc) (1:100) together with a secondary biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Vectastatin®) were used as described in **Section 3.3.3.1 and 3.3.3.2**.

6.3.1.1.2 Nitrotyrosine immunoblotting (3 NT)

An identical technique as described for XOR immunoblotting was used to quantify the relative amounts of 3 NT within aortic tissue (**Section 3.5**). Specimens were prepared as described in **Section 3.4.1** and total protein estimated using the Bradford assay (**Section 3.4.2**). Twenty micrograms of total protein was loaded per well. Membranes were probed with a rabbit anti-nitrotyrosine (Upstate biotechnology, 1:1000), followed by a conjugated horseradish peroxidase swine-anti rabbit secondary antibody used at 1 in 2000 dilution (Dako, 1:2000). Each sample was analysed in triplicate. Radiographic films were digitally imaged and stored as TIFF files. The mean pixel intensity at each

well position was measured as described in **Section 3.5.1**. Relative densitometry of control tissue and aneurysm tissue were compared.

6.3.1.2 Lucigenin-enhanced chemiluminescence to detect superoxide generation

Superoxide levels were measured by lucigenin-enhanced chemiluminescence as previously described in **Section 3.7.3**

6.3.1.2.1 Sample preparation

Samples were prepared in one of three ways,

1. Samples were homogenised in the standard homogenisation buffer as described in **Section 3.4.1.**
2. Fresh samples were crushed in liquid nitrogen and homogenised in 1xPBS. Samples were centrifugation at 4000 rpm for 10 minutes and supernatants retained for subsequent experiments.
3. Fresh samples were crushed in liquid nitrogen and homogenised in collagenase Type II (1mg/ml) in PBS/1% Tween (1ml/100 mg wet weight). Samples were centrifuged at 4000 rpm for 10 minutes and supernatants retained for experiments.

6.3.1.2.2 Assay protocol

An aliquot of homogenisation supernatant (50µl) was placed in a well and 10µl of 2mM DTPA and 40µl PBS were added. Lucigenin was made up to a stock concentration of 2mM in PBS and NADH made up to a stock concentration of 2mM in PBS. The reactions were initiated by the automated injection of 50µl of substrate NADH followed by 50µl of lucigenin into the wells of a 96-well plate. Measurements were as described in **Section 3.7.3**. Each sample was analysed in triplicate and the assay repeated three times. The results are expressed in arbitrary light units (ALU).

6.3.1.3 Controls and sample size

AAA tissue was compared to non-AAA derived aortic tissue (Controls). Control aortic tissue was obtained from cadaveric organ donors from the cuff on the renal artery/aorta. No formal power calculation could be undertaken to calculate the number of samples required in either the AAA group or control group. Previous published studies using tissue homogenates that have showed a difference between AAA and AOD/normal tissue, have all included small numbers of control samples, ranging between 3 and 10 . In view of this, the aim was to obtain 10 'normal' non-AAA tissue samples.

6.3.2 To determine if oxidant stress increases the activation of pro-MMPs

To determine if reactive oxygen species activate MMP-2 or MMP-9, commercially available purified MMP-2 was incubated with a superoxide generating system.

Purified Human MMP-2 and MMP-9 (Chemicon) are 90% pro-enzyme and as such only give low gelatinase activity.

6.3.2.1 Standard curve

Solution of 300ng/ml of MMP-2 or MMP-9 were prepared and a standard curve generated using a serial dilution of purified MMP-2/9 activated with AMPA as described in the literature provided with the Chemicon gelatinase kit (*Appendix*).

6.3.2.2 Experimental samples

Eighty microlitres of a 360 ng/ml MMP solution were added per well to a 96 well plate. Xanthine oxidoreductase (Biozyme) (Log 10 scale 0.001- 1mu) in the presence of Hypoxanthine (Final 20 μM) was used to generate superoxide. The total reaction volume was 100 μl, and was made up with 1x PBS. Samples were incubated for 3 hours at 37°C. Each reaction was performed in triplicate and the assay was repeated three times. Xanthine oxidoreducatase and hypoxanthine reaction blanks were used. Specificity for XOR was confirmed with the addition of 100mM allopurinol.

6.3.2.3 Experimental end-points

Gelatinase (elastolytic) activity after incubation was assessed using the Chemicon gelatinase assay as described in **Section 3.11.2** and the succinylated elastin assay as described in **Section 3.11.3**.

6.3.2.4 Experimental number

No formal power calculation could be undertaken for this experiment as no previous studies have been reported. The experiment was carried out in triplicate for each experimental condition and repeated 3 times (N=3)

6.3.3 Statistics

Statistical analysis was performed using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Data were assessed for deviations from Gaussian distribution using the Kolmogorov-Smirnov test. Continuous variables were expressed as means ± Standard error of the mean (SEM) for parametric data. Variables were analysed with students' t-test or One-way analysis of variance (ANOVA) for multiple group comparison. Statistical significance was considered for p values less than 0.05.

6.4 Results

6.4.1 Demonstration of oxidant stress in aortic tissue

6.4.1.1 Peroxynitrite

6.4.1.1.1 Nitrotyrosine immunolocalisation (3 NT)

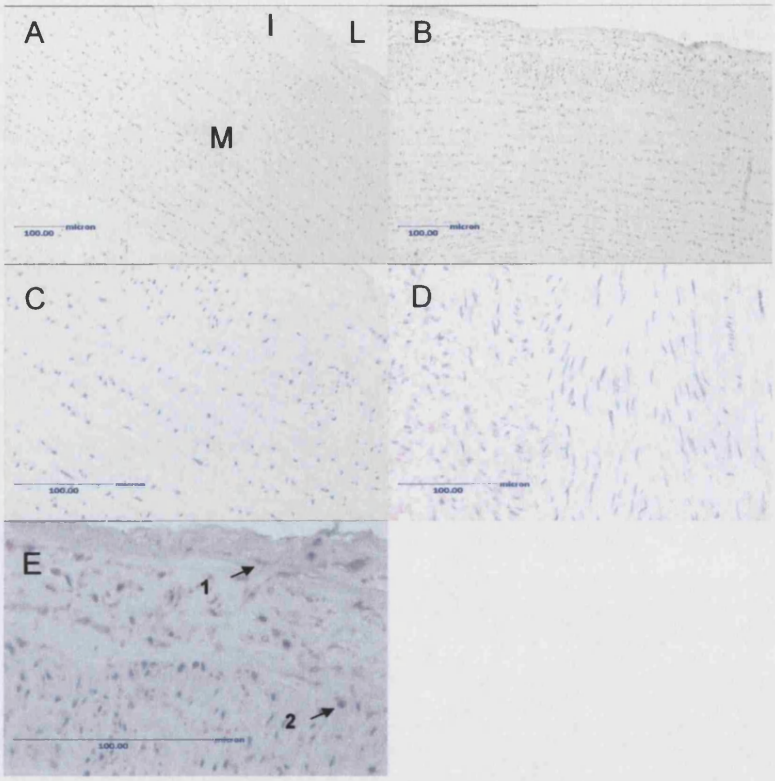

Figure 6-1 Photomicrographs showing the immunolocalisation of nitrotyrosine (red) in human non-AAA tissue

Specimens were probed with a anti-nitrotyrosine (Upstate Biotechnology Inc) (1:100) and a biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Vectastatin®). Slides a and c are negative controls. Slide b (x50) shows diffuse immunopositivity for nitrotyrosine throughout the artery wall. Image d (x 200) and image e (x 400) show both extracellular (1) and intracellular (2) positivity for 3NT. *(L=Lumen, M=Media, Ad=Adventitia).*

Immunopositivity for nitroyrosine was seen throughout the aortic wall (B) with both the intima (I) and media showing positivity for 3 NT. Immunopositivity was particularly prominent in the intima and inner media. Interestingly the distribution of immunopostivity was similar to XOR which is a known potential source of peroxynitrite (*Godber et al. 2000a*).

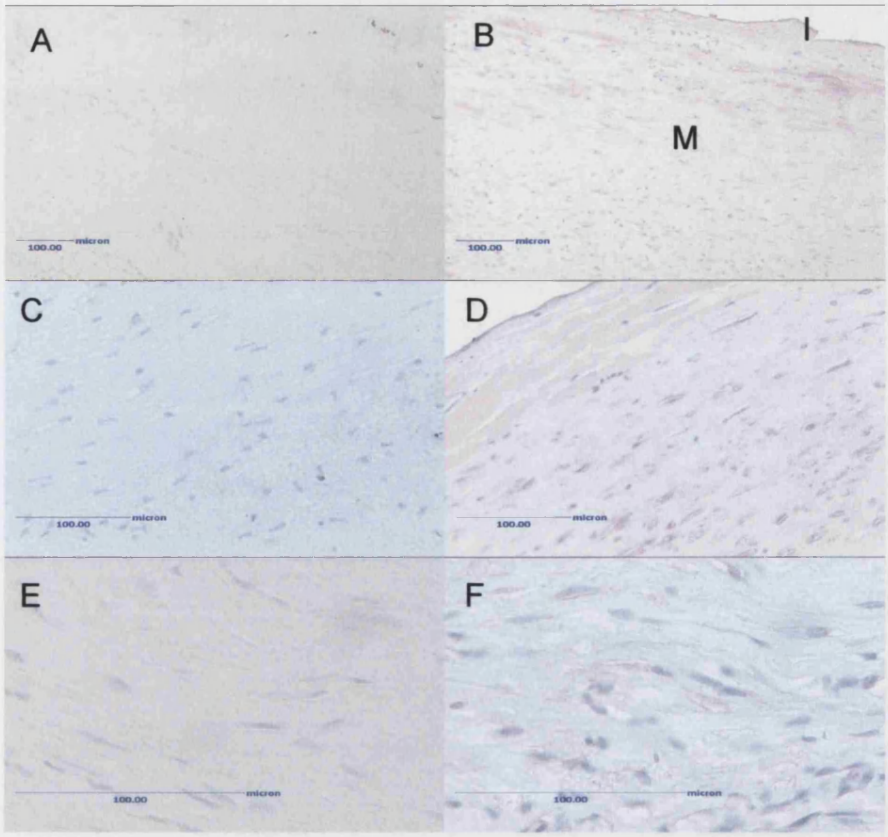

Figure 6-2 Photomicrographs showing the immunolocalisation of nitrotyrosine (red) in human AAA tissue.

Specimens were probed with a anti-nitrotyrosine (Upstate Biotechnology Inc) (1:100) and a biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Vectastatin®). Slides a, c and e are negative controls. Slide b (x50) shows diffuse immunopositivity for nitrotyrosine throughout the artery wall. Image d (x 200) and image f (x 400) show both extracellular and intracellular positivity for 3NT. *(L=Lumen, M=Media, Ad=Adventitia).*

The pattern and distribution of immunopositivity for nitrotyrosine was similar in AAA tissue as in non-AAA derived aortic tissue.

6.4.1.1.2 Nitrotyrosine immunoblotting (3 NT)

The amount of nitrotyrosine in AAA samples was compared to nitrotyrosine levels in non-aneurysm control tissue using a semi-quantitative immunoblot technique. Staining with Amido black confirmed equal and complete transfer of protein to the nitrocellulose paper. Blank wells were loaded with 1x PBS. Each sample was analysed in triplicate and the assay repeated twice. These data represent the mean value of two separate analyses (AAA n = 18, Controls n =13). Results are expressed in relative densitometric units.

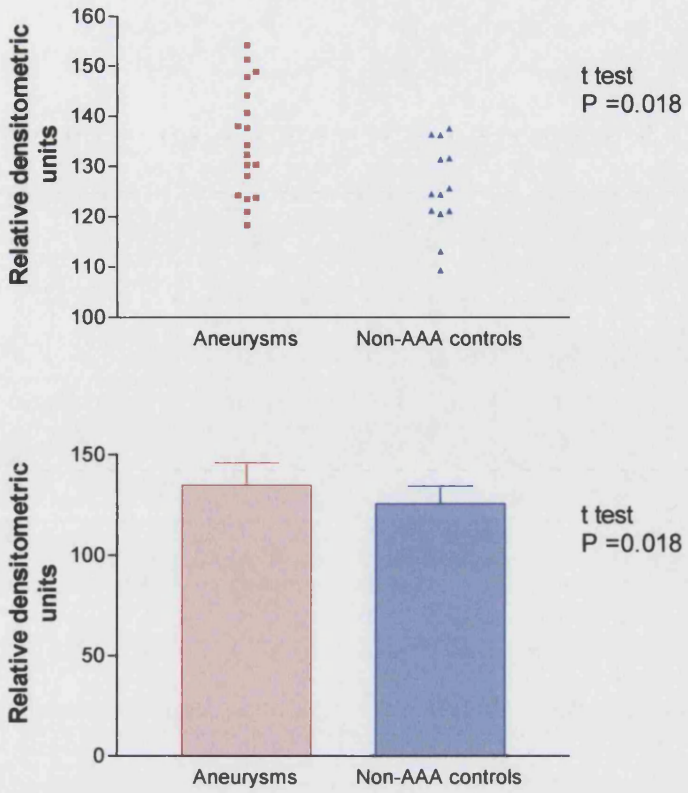

Figure 6-3 Nitrotyrosine protein levels in aortic tissue quantified by immunoblot

Results expressed in relative densitometry units. AAA (n=18) and control samples (n=13). Data are mean ± SD of sample triplicates analysed twice.

The data demonstrate that significantly more 3 –NT is detected in AAA tissue compared to aortic tissue from control patients.

The result was confirmed by calculating the nitrotyrosine/protein ratios in order to confirm that slight variations in protein transfer did not account for the observed statistically significant difference. Each sample protein density was measured by taking a digital image after Amido black staining, which was then analysed using Scion imaging software.

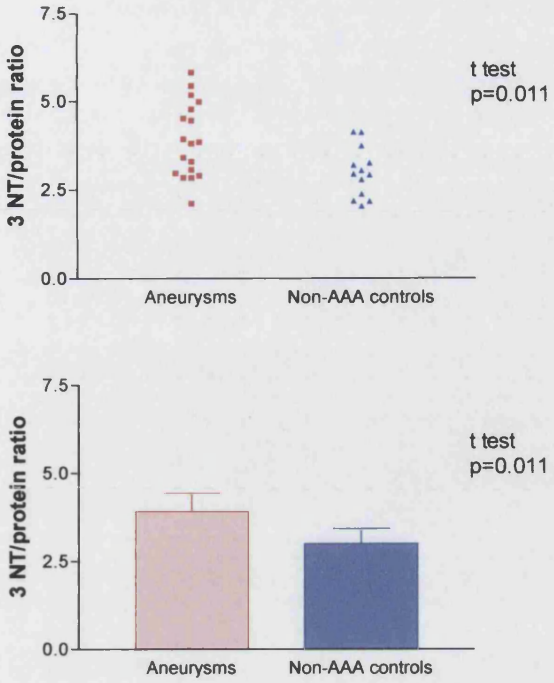

Figure 6-4 Nitrotyrosine protein levels quantified with immunoblot (expressed as 3NT/total protein)

Correction for total protein content in each sample also showed statistical significance confirming the validity of the observation.

6.4.1.2 Lucigenin-enhanced chemiluminescence

6.4.1.2.1 Samples in homogenisation buffer

Frozen samples prepared by homogenisation with standard homogenisation buffer (**Section 3.4.1**) were analysed. Sixteen AAA samples were compared to 8 non-AAA control samples. Ten micrograms of bovine XOR (Biozyme) was used as a positive control. Reaction blanks contained 1x PBS. Blank recordings were subtracted from sample values. Each sample was analysed in triplicate and the assay repeated three times. The data represent the mean of three assays. Results are expressed in arbitrary light units per milligram of total protein (ALU/mg total protein). **Figure 6.5** shows that AAA and control samples contained no measurable activity using NADH as a substrate.

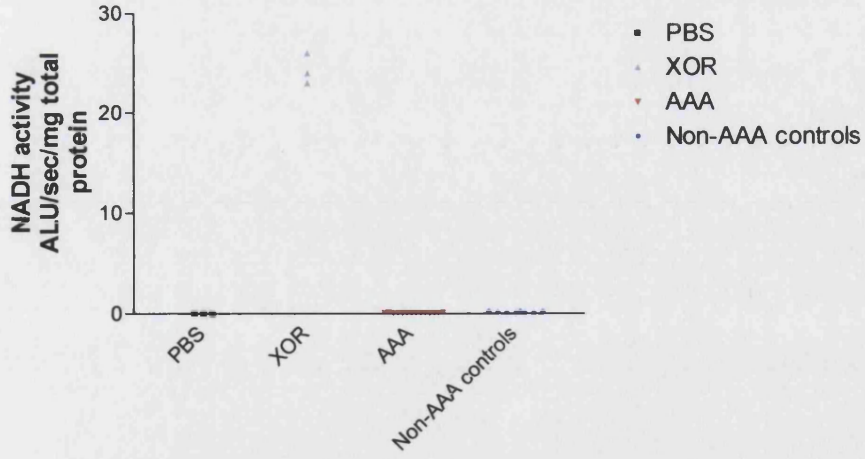

Figure 6-5 Chemiluminesence activity detected in aortic tissue using NADH as substrate

AAA (n=16), control tissue (n=8)

6.4.1.2.2 Samples prepared in liquid nitrogen/PBS

5 AAA samples and 4 control samples were analysed. Ten micrograms of bovine XOR (Biozyme) was used as a positive control. Reaction blanks contained 1x PBS. Blank recordings were subtracted from sample values. Each sample was analysed in triplicate and the assay repeated three times. The data represent the mean of three assays. **Figure 6.6** shows that AAA and control samples contained no measurable activity using NADH as a substrate.

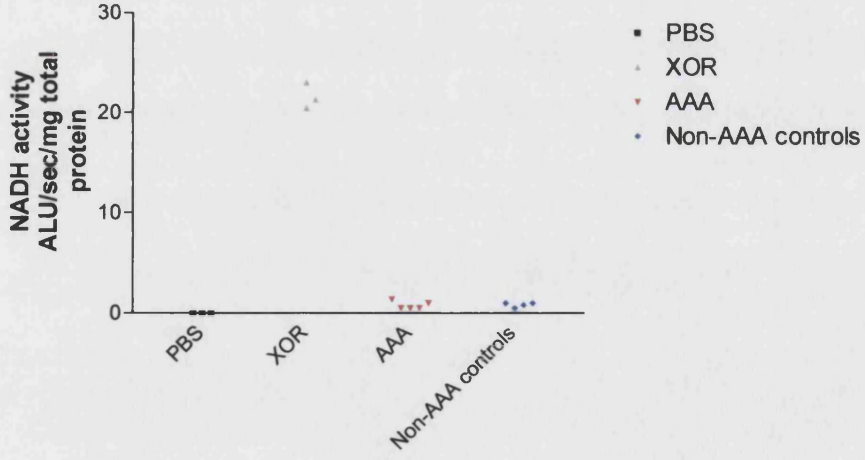

Figure 6-6 Chemiluminesence activity detected in aortic tissue using NADH as substrate.

AAA (n=5), control tissue (n=4). Samples prepared in liquid nitrogen/PBS

6.4.1.2.3 Samples prepared in liquid nitrogen/collagenase type II

7 AAA samples and 4 control samples were analysed. Ten micrograms of bovine XOR (Biozyme) was used as a positive control. Reaction blanks contained 1x PBS. Blank recordings were subtracted from sample values. Each sample was analysed in triplicate and the assay repeated three times. The data represent the mean of three assays. **Figure 6.7** shows that AAA and control samples contained no measurable activity using NADH as a substrate.

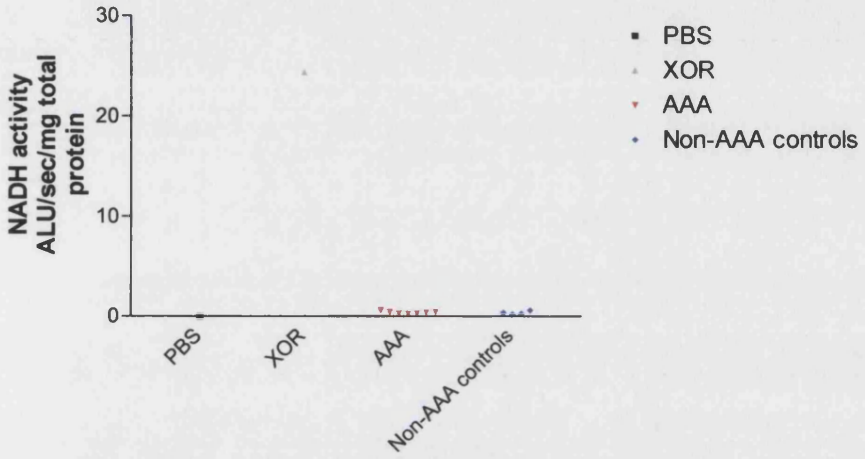

Figure 6-7 Chemiluminesence activity detected in aortic tissue using NADH as substrate.

AAA (n=7) or control tissue (n=4). Samples prepared in liquid nitrogen/collagenase

6.4.2 Oxidant stress and MMP activation

6.4.2.1 MMP-2

A standard curve was generated using purified MMP-2 activated with AMPA as described in the protocol provided with the Chemicon gelatinase kit. Samples were incubated for 3 hours at 37°C. Each sample was analysed in triplicate and the assay repeated three times.

The results show that the combination of XOR and hypoxanthine (20μM) resulted in increasing activation of purified MMP-2 as detected by the Chemicon gelatinase activity assay. The reduction in activation seen by 100μM Allopurinol (an XOR inhibitor) confirms that the activation is XOR dependent.

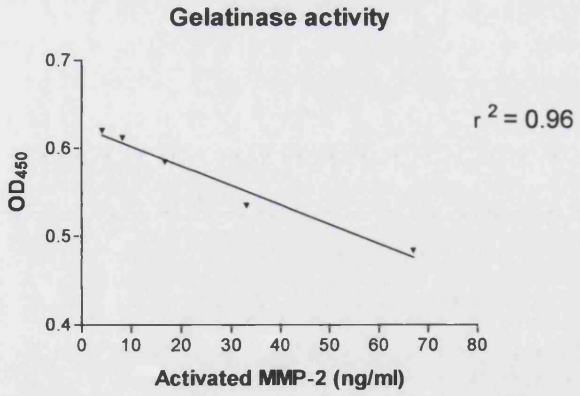

Figure 6-8 Standard curve for APMA activated MMP-2 in the gelatinase activity assay (Chemicon)

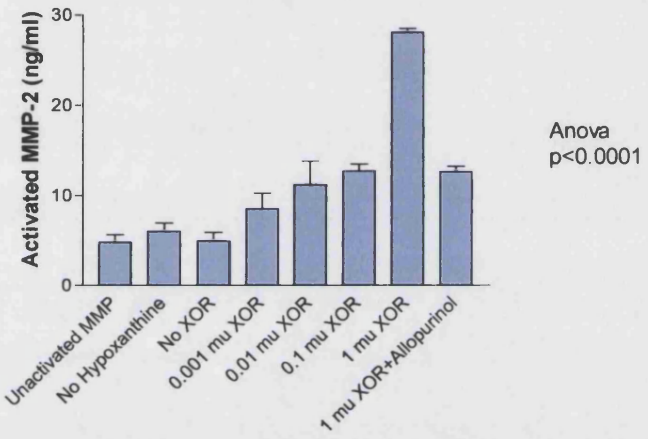

Figure 6-9 MMP-2 activation with hypoxanthine (20µm) and /XOR (0.001 mu-1 mu XOR)

Activity was measured with the gelatinase activity assay (Chemicon). Data are mean ± SEM of sample triplicates analysed three times

The experimental samples were also analysed using the succinylated elastin assay as described in **Section 3.11.3**. A standard curve was generated using AMPA activation of serial dilutions of purified MMP-2 as described previously. Adequate succinylation of gelatin was confirmed by incubating succinylated gelatin with 50µl of 0.03% TBNSA solution and recording absorbances at 450nm.

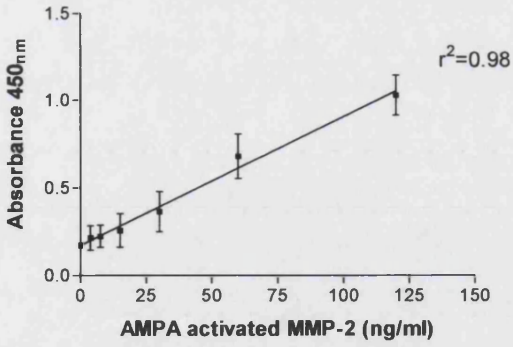

Figure 6-10 Standard curve for AMPA activated MMP-2 determined using the succinylated gelatin assay

Each reaction contained 15µl of substrate, 40µl of experimental sample and 95µl of buffer (PBS/$CaCl_2$ (1mM)). Specificity of the assay for gelatinase (MMPs) was confirmed with the addition of 10mM EDTA to the assay buffer. Reactions were incubated for 30 mins at 37^0C. Fifty micolitres of 0.03% TBNSA solution was added to each reaction and samples incubated at room temperature for 20 minutes.

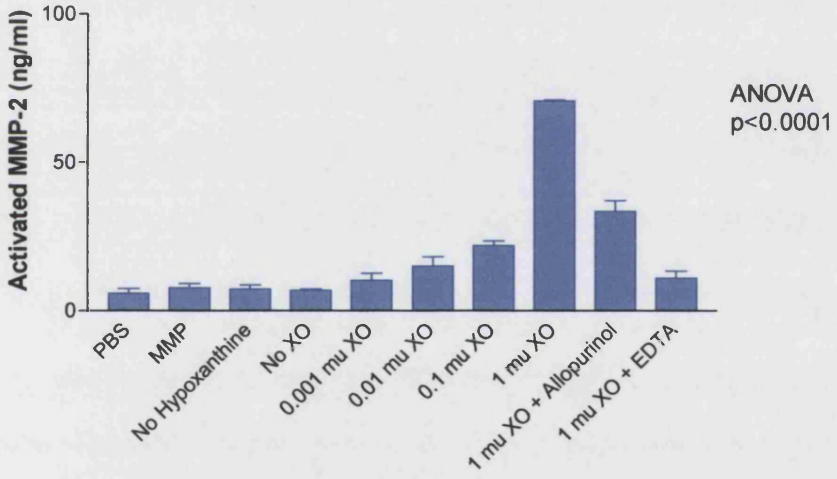

Figure 6-11 MMP-2 activation with hypoxanthine (20µm) and /XOR (0.001 mu-1 mu XOR) Activity was measured with the succinylated gelatin assay.

Data are mean ± SEM of sample triplicates analysed three times

The results from the succinylated gelatin assay confirm the activation of purified MMP-2 with XOR/hypoxanthine. The reduction in activated MMP-2 seen with the addition of allopurinol (100µM) confirms that XOR activity is responsible for MMP-2 activation. The reduction in activated MMP-2 seen with EDTA (10mM) in the assay buffer confirms the specificty of this assay for gelatinases (MMPs).

6.4.2.2 MMP-9

A standard curve was generated using purified MMP-9 activated with AMPA as described previously.

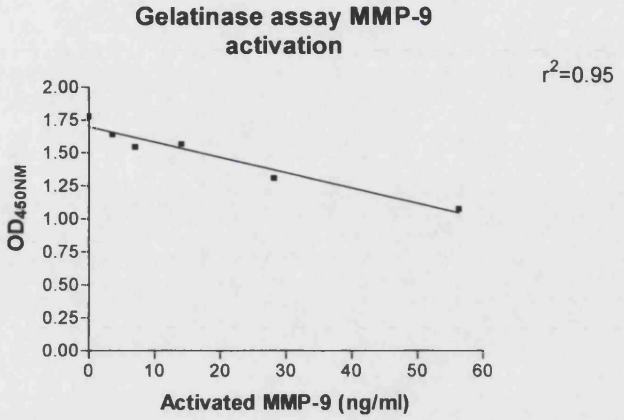

Figure 6-12 Standard curve for AMPA activated MMP-9 determined using the succinylated gelatin assay

Samples were incubated for 3 hours at 37°C. Each sample was analysed in triplicate and the assay repeated three times. Experimental samples were only analysed with the succinylated gelatin assay. The results show that the combination of XOR and hypoxanthine (20μM) resulted in increasing activation of purified MMP-9. The reduction in activation seen by 100μM Allopurinol (an XOR inhibitor), confirms that this activation is XOR dependent.

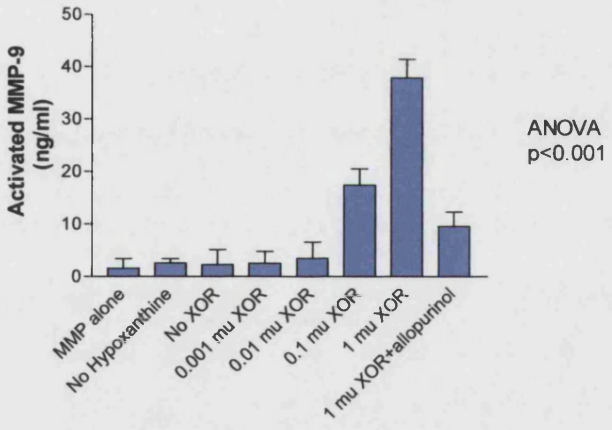

Figure 6-13 MMP-9 activation with hypoxanthine and XOR

Activity was measured with the succinylated gelatin assay. Data are mean ± SEM of sample triplicates analysed three times

6.5 Discussion

Oxidant stress could promote aneurysm formation through increased expression and activation of the MMP cascade. It is known that reactive oxygen and nitrogen species are produced by the 3 major types of cells resident in the aortic wall, namely VSMCs, fibroblasts and endothelial cells as well as by infiltrating macrophages and in addition oxidant stress has been reported to activate MMPs under *in vivo* conditions (*Rajagopalan et al. 1996; Siwik et al. 2001; Miller et al. 2002*).

Since the conduction of our study, one published report has appeared in the literature concerning oxidant stress in AAAs. In this study, segments of AAA tissue were compared to adjacent non-aneurysmal tissue within the same patient. This study found that superoxide levels (measured by lucigenin-enhanced chemiluminescence) were higher in the AAA segments compared with the adjacent non-aneurysmal aortic segments. The increased superoxide was associated with the inflammatory infiltrate and Dihydroethidium staining indicated that that the increased superoxide was localised to inflammatory cells and VSMCs. Further immunostaining for $p47^{phorx}$ and $p22^{phorx}$, two of the subunits of NAD(P)H oxidase and the fact that lucigenin enhanced chemiluminescence could be attenuated by treatment with DPI (diphenylene iodonium) a flavin inhibitor suggested that an NAD(P)H oxidase was an important source of this superoxide. Formation of thiobarbituric acid-reactive substances and conjugated dienes, two indices of lipid peroxidation, were also increased in AAA compared with non-aneurysmal segments. In addition enhanced nitrotyrosine immunostainning, a marker for amino acid oxidation induced by several oxidant species, including peroxynitrite, the highly reactive product of superoxide and nitric oxide, was enhanced in AAA tissue compared with non-AAA segments (*Miller et al. 2002*). The results from this study all suggest that oxidant stress was associated with AAA formation (*Miller et al. 2002*). An important point made by Miller, which is relevant to any conclusions drawn from these data as well as from this study, is that the investigations were performed on AAA at an advanced stage (end-staged disease), and the importance of such oxidant stress in the development of smaller sub-clinical aneurysms is unknown.

This study using aortic tissue derived from AAA patients and control tissue from non-AAA aortas, which unfortunately were not age matched, demonstrates increased nitrotyrosine in AAA tissue. Immunoblotting showed statistically significantly more peroxynitrite present in AAA tissue compared to normal controls. Adjusting for total protein to eliminate small variations in protein transfer made no difference to this result. Despite the statistical difference between the two groups using a semi-quantitative

technique, the clinical significance of this small difference between the two groups is uncertain. As far as I am aware there is no quantitative technique to measure peroxynitrite in tissue (*Tarpey et al. 2001*). These data for peroxynitrite support the observation of Miller (*Miller et al. 2002*).

Attempts to measure superoxide production from aortic tissue using Lucigenin enhanced chemiluminescence were difficult and frustrating. Despite several alternative techniques of sample preparation, no activity could be detected. This either means that no activity is present or it is below the detection limit of this assay. These results, together with the problems encounted with XOR activity measurement suggest that sample storage/preparation may have been a problem. In Miller's recent paper on oxidant stress, published after the conclusion of my time in the laboratory, superoxide levels measured by lucigenin-enhanced chemiluminescence were detectable. In this paper, aortic segments were placed in 0.9% saline (4°C) and transported to the laboratory. Aortic segments were placed in PBS and lucigenin (5μmol/L), and after 2 minutes of dark adaptation, relative light units (RLU) emitted were measured for 5 minutes after NADH (0.1mmol/L) or NADPH (0.1mmol/L) was added to the PBS-lucigenin containing the vessel segment. Some segments were pre-incubated with polyethyleneglycolated superoxide dismutase (SOD, 250 U/mL) or the flavin inhibitor diphenylene iodonium (DPI, 0.1 mmol/L). Surface areas were measured for each segment to allow normalization for tissue size (*Miller et al. 2002*). Our technique differed in the following respects;

1. Samples were stored in Hanks balanced salt solution
2. Samples were homogenised and supernatants used for experiments
3. The concentration of Lucigenin was 100x greater in our technique
4. The concentration of NADH was 5x greater in our technique

MMPs are secreted in a latent zymogen form in which the prodomain shields the catalytic site. This conformation of zymogens is maintained due to thiol interactions between cysteine residues in the prodomain and the zinc atom present in the catalytic site (*Ye et al. 1998*). Reactive oxygen species are known to react with thiol groups such as those involved in preserving MMP latency, and several previous reports have demonstrated that exogenous generated reactive oxygen and nitrogen species activate MMP-2 and MMP-9 in isolated animal cell culture experiments (*Rajagopalan et al. 1996; Belkhiri et al. 1997; Inoue et al. 2001; Siwik et al. 2001*).

I decided to investigate the possible role of oxidant stress on the activation of purified MMP-2 and MMP-9 in cell free experiments to determine if oxidant stress activated

human MMP. The results from these experiments demonstrate that purified human MMP-2 and MMP-9 could be activated by superoxide generated from hypoxanthine/xanthine oxidase.

The limitations that I have about extrapolating these data are;

1. Whether this degree of oxidant stress exists in *in vivo*. There is only one report of oxidant stress in AAA tissue (*Miller et al. 2002*). Whilst data from our study show evidence of peroxynitrite, we were unable to measure superoxide in aortic tissue.
2. We have only measured superoxide on MMP-2 and MMP-9 activation. The effects of other reactive species on MMP-2 and indeed the effects of reactive species on other MMPs are unknown.

Tissue derived from patients with AAAs have been shown to have reduced levels of ascorbic acid, vitamin E and other anti-oxidant enzyme activities (*Sakalihasan et al. 1996b; Dubick et al. 1999*). These observations combined with our data and that reported by Miller would add evidence to a hypothesis that oxidant stress within the wall of the aorta could have a potential role in the pathogenesis of AAA.

Interestingly recent research, which has focussed on potential mechanisms to decrease AAA formation and expansion can all be potentially explained via oxidant stress related mechanisms. Simvastatin, an HMG-CoA reductase inhibitor has been shown to decrease MMP secretion and decrease active MMP-2 in aortic wall explants (*Bellosta et al. 1998; Marz et al. 2000; Evans et al. 2002*). One such mechanism by which it may act is by inhibition the redox sensitive transcription factor NFκB that increases MMP expression (*Bond et al. 2001*).

Angiotension II, a pro-inflammatory mediator has been shown to induce AAA formation in hypercholesterolaemic mice (*Daugherty et al. 2000*). It is believed that angiotensin II stimulates the production of reactive oxygen species via NAD(P)H oxidase in VSMCs (*Griendling et al. 1994*). Recent evidence has indicated that treatment with Angiotensin converting enzyme inhibitors (ACE inhibitors) suppresses the formation of angiotensin II and the subsequent development of elastase-induced AAAs in rats (*Liao et al. 2001*). It could be postulated that such effects on AAA development are due to the decrease in angiotensin II and subsequent decreased in oxidant stress.

DISCUSSION

Chapter 7 General discussion

7.1 Summary

The hypothesis that was tested is summarised in **Figure 7.1**.

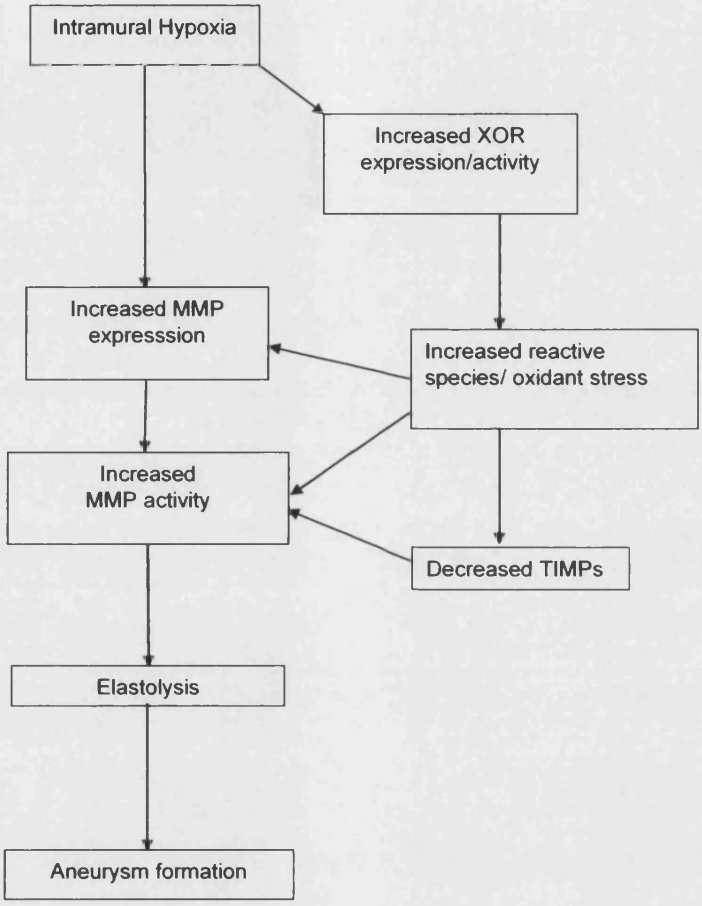

Figure 7-1 Diagrammatic representation of hypothesis.

7.1.1 Xanthine oxidoreductase

In order to determine if XOR had a role in the pathogenesis of AAAs, end-stage AAA tissue was compared to non-AAA control tissue obtained from cadaveric organ donors. Unfortunately the controls and AAA tissue were not from age-matched patients, control tissue was from a statistically younger population. Whilst XOR protein could be demonstrated in aortic tissue, localised to the media and intima, there was no difference in XOR protein levels between AAA patients and controls using both an ELISA and immunoblotting technique. In addition no XOR activity could be detected in aortic tissue (AAA and controls). The conclusion from this work is that there is no difference

in either the expression or activity of XOR between AAA and control tissue, and thus it is unlikely that XOR has a role in AAA formation

The limitation of this study that particularly needs to be pointed out is that it relies on the use of end-stage tissue, and thus no conclusions can be drawn as to whether XOR has a role in early AAA formation.

7.1.2 Hypoxia and elastolytic activity

The central theme that hypoxia leads to increased elastolytic activity was tested in VSMCs culture experiments. Vascular smooth muscle cells (VSMCs) were chosen as the most appropriate cell type as these cells have been shown previously to be a source of elastolytic MMPs. VSMCS were isolated from aortic explants and exposed to hypoxic. These experiments did not demonstrate any increase in MMP-2 or MMP-9 expression, MMP-2 and MMP-9 protein level, or elastolytic activity in cells exposed to hypoxia. In addition hypoxia had no effect on XOR expression or protein levels in VSMCs exposed to hypoxia. These studies effectively proved that the hypothesis was incorrect and no relationship between hypoxia and elastolytic activity could be established.

The limitations of these particular studies were as follows;

1. No measurement of hypoxia was undertaken in *in vivo* conditions to establish the degree of hypoxia present.
2. Only one degree of hypoxia was used in the experiments. As to whether this is the level that exists in either physiological situations or pathological conditions such as in the wall of the AAA is not known. The effect of hypoxia was used in a situation in which it could be argued does not represent *in vivo* conditions.
3. The use of isolated cell culture for experiments is open to criticism.
4. No MMP-9 mRNA or MMP-9 protein was detectable in the experiments.

7.1.3 Oxidant stress and elastolytic activity

This work was an adjunct to the original hypothesis but seemed to be logical continuation. I postulated that increased oxidant stress (increased reactive oxygen and nitrogen species) was involved in AAA formation. Despite the demonstration of increased levels of peroxynitrite in AAA tissue compared to control tissue, I was unable to demonstrate any differences in the ability to generate superoxide which could be viewed as an index of raised oxidant stress in tissue.

In–vitro experiments with commercially available MMP-2 and MMP-9 confirmed that superoxide activated these elastolytic enzymes. The conclusion from these studies was

that there was some evidence of raised oxidant stress within AAA tissue and that such reactive species can activate pro-MMPs leading to increased elastolytic activity.
The limitation of this study is the obvious concern that I was unable to measure superoxide generating activity within the samples. In addition it is not known whether the degree of oxidant stress that was used in the *in vitro* experiments exists in the *in vivo* situation.

7.2 Future work

7.2.1 Xanthine oxidoreductase

I do not feel that any further work analysing XOR protein in aortic tissue is likely to be beneficial. However, previous reports of increased uric acid in tissue from AAAs suggests that the activity of XOR in AAAs may be increased, although I have been unable to prove that in this study (*Patetsios et al. 1996; Patetsios et al. 2001*). Perhaps alternative techniques of measuring XOR activity, including high performance chromatography could be utilised to determine if XOR activity is increased in AAA tissue (*Patetsios et al. 1996*).

7.2.2 Hypoxia

The paper published by Vorp suggests that localised areas of hypoxia exist in AAA tissue (*Vorp et al. 2001*). The data from my work would suggest that if hypoxia does have a role in the pathogenesis of aneurysm formation then it is not by directly increasing MMP expression and elastolytic activity in resident VSMCs.
Vorp demonstrated that the AAA wall adjacent to a thick layer of ILT has localised areas of hypoxia.and these areas were associated with increased localized mural neovascularisation and inflammation and regional wall weakening (*Vorp et al. 2001*). Whilst this study demonstrates hypoxia in the wall of human aortic aneurysms it does not answer the question as to whether hypoxia is a primary aetiological event or a secondary consequence. The question as to whether hypoxia exists in non-aneurysmal vessels or in the early stage of aneurysm development remains unanswered.
In view of the findings in this study and the work by Vorp, I believe the following maybe useful areas of further research

1. The development and use of a non-invasive assessment of intra-mural hypoxia would greatly aid this area of research. This would then allow the oxygen tension areas to be measured in the aorta of non-AAA patients and in patients with small AAAs without the ethical concerns associated with invasive assessment

2. Vorp's data suggest that hypoxia is associated with inflammation and neovascularisation, and it may be down this pathological pathway that hypoxia has a role in the development of AAAs. This probably merits further research.
3. The effect of differing oxygen tensions on collagenolytic MMPs, which are believed to be important in AAA rupture could be investigated. Laminated intra-luminal thrombus is found in increasing amounts as AAA expand and has been demonstrated to be associated with localised areas of hypoxia (*Adolph et al. 1997; Vorp et al. 2001*). Perhaps the build up of thrombus as AAAs expand leads to hypoxia which activates the collagenolytic MMPs that lead to AAA rupture?
4. The effect of hypoxia on elastolytic MMPs could be assessed in an alternative model of AAA formation such as the aortic tissue ex-vivo explants (*Wills et al. 1996*). The potential advantage of this model over isolated cell culture experiments is that it does not eliminate the paracrine influence of other cell types and is thus a better model of *in vivo* conditions.

7.2.3 Oxidant stress

I believe that this area of research is probably going to be the most productive. The initial data from this study in combination with previous reports suggest that oxidant stress is increased in AAA tissue (*Sakalihasan et al. 1996b; Dubick et al. 1999; Miller et al. 2002*). In addition, evidence exists to suggest that increases in oxidant stress are associated with alterations in MMP expression, MMP protein levels and MMP activation that lead to increased elastolysis and collagenolysis (*Rajagopalan et al. 1996; Galis et al. 1998; Maeda et al. 1998b; Buhimschi et al. 2000; Siwik et al. 2001; Uemura et al. 2001*).

Future areas of research would be as follows

1. Measurement of oxidant stress in tissue obtained from AAA could futher be compared to tissue obtained from non-AAA controls. Alternative techniques to measure oxidant stress such as formation of thiobarbituric acid-reactive substances and conjugated dienes could be used.
2. Measurement of the degree of oxidant stress in plasma of patients with AAAs could be compared to age and gender matched controls. The ideal population for this study would be a screening cohort of patients, such as in the Gloucestershire screening programme. In addition, rates of expansion of AAAs could be correlated with the degree of oxidant stress to determine if oxidant stress could explain variations in expansion commonly seen in AAA progression.

3. The effect of experimental oxidant stress on MMP expression, MMP protein or elastolytic or collagenolytic activity could be assessed in a model of AAA. The alternative models that could be used for this study would be either isolated tissue culture or whole tissue aortic explants from either AAA or control non-AAA patients. End-points would be MMP mRNA levels, MMP protein levels measured by commercially available ELISA and net elastolytic activity that colud be measured by either commercially available assays or the succinylated gelatin assay.

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APPENDIX

Shandon Hypercenter II tissue processing apparatus

The procedure for processing tissue samples into wax blocks

	Temp (°C)	Immersion time
Formalin (10%)	RT	10mins
Alcohol (70%)	RT	30mins
Alcohol (80%)	RT	1hr
Alcohol (95%)	RT	1hr
Alcohol (absolute)	RT	1hr
Alcohol (absolute)	RT	1hr
Alcohol (absolute)	RT	1hr
Xylene	RT	1hr
Xylene	RT	1hr
Xylene	RT	1hr
Wax	60	1hr
Wax	60	1hr

Alcohol = Industrial methylated spirits.

Slides

Poly-l-lysine (Sigma Cat. No. P1524). 0.05-0.1% Poly-l-lysine MW>35000 in DDW
Microscope slides (BDH. Cat. No. 406/0184/02)

Histological procedures

Dewaxing slides in xylene and IMS

1. Deparaffinize tissue, 2x 30 seconds in Xylene (BDH, Cat. No. 30578564)
2. Fix in IMS, 2 x 30 seconds (BDH, Cat. No. 30244)
3. Rehydrate in 1x PBS for 5mins.

Haematoxylin and eosin staining technique

1. Tap water; 5 minutes
2. Harris' haematoxylin; 5 minutes
3. Acid alcohol; 1 minute
4. Tap water; 5 minutes
5. Eosin; 30 seconds
6. Water; 5 minutes
7. IMS; 30 seconds
8. IMS; 30 seconds
9. Xylene; 30 seconds (100%)
10. Xylene; 30 seconds (100%)
11. Mount DPX

Immunohistochemistry

Xanthine oxidase (Paraffin-Embedded sections)

1. Dewax slides and rehydrate in 1x PBS
2. Blocking solution. Incubate in wet box with 200µl per slide blocking solution for 30 minutes.
   5 mg bovine serum albumin (BSA),
   333µl normal goat serum (Sigma No G9023)
   10 ml PBS
3. Wash. PBS, 1 x 5 minutes
4. Primary antibody. Incubate with 200µl per slide primary antibody overnight at 4°C.
   1:50 – 1:200 dilution of antibody in blocking solution
   Polyclonal rabbit anti-XOR. (Chemicon AB 1242)
5. Wash. PBS, 2 x 5 minutes
6. Secondary antibody. Incubate with 200µl per slide secondary antibody for 30 minutes
   1:200 dilution of antibody in blocking solution
   1 drop of blue bottle in 10 ml (1x) PBS (Biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Vectastatin® ABC-AP reagent, No AK –5001, Peterborough UK))
7. Wash. PBS, 2 x 5 minutes
8. Application of the avidin-bound enzyme probe to link to the biotinylated secondary antibody
   2 drops of A (Avidin) bottle and 2 drops of B (biotinylated alkaline phosphatase) bottle to 10 ml 1x PBS (Vector Laboratories, Vectastatin® ABC-AP reagent, No AK –5001, Peterborough UK),200µl per slide
   Incubate for 30 minutes
9. Wash. PBS 2 x 5 minutes
10. Application of chromogenic enzyme substrate. Naphthol AS -MX phosphate/Fast red TR Fast™ (Sigma. Cat. No. F-4523).
    10 ml distilled water
    Ultra-filtration
    200µl per slide
11. Stop reaction with cold water
12. Conterstain with Mayer's Haematoxylin for 1 minute
13. Rinse in running tap water for 5 minutes
14. Mount with 4 drops of Aquamount per slide.

Primary antibody for detection of 3 nitrotyrosine

Identical to the technique for XOR immunohistochemistry except primary antibody was rabbit anti-nitrotyrosine (Upstate Biotech, cat no: 06-284)

Tissue experiments

Hanks balanced salt solution (HBSS)

This contained $Ca^{2+}$and Mg^{2+}, sodium bicarbonate and phenol red and was sterile. (Gibco, Cat. No 2402 091)

Homogenisation buffer

50mM KPO_4 pH 7.4 (Sigma. Cat. No. P0662)
1mM EDTA (Promega, UK. Cat. No. H503a)
1mM PMSF (Sigma. Cat. No. P-7626)
Pepstatin A (1µg/ml) (Sigma. Cat. No. P4265)
Antipain (1µg/ml) (Sigma. Cat. No. A-6191)
Leupeptin (1µg/ml) (Sigma. Cat. No. L-2023)

Protein assay

Bio-Rad Protein Assay Kit II (Bio-Rad, UK. Cat. No. 500-002)

Western blotting

2x loading buffer

50mM Tris-HCL pH 6.8 (Sigma Cat. No. T3253)
100mM DTT (Promega Cat. No. V3151)
2% SDS (Sigma Cat. No. L-3771)
20% Glycerol (Sigma Cat. No. G7893)
Added to this is 0.01% bromophenol blue (Promega Cat. No. H5011)

Rainbow markers

High molecular weight range (14300-220000). (Amersham, UK. Cat. No. RPN 756)

Resolving gel (8%) (10mls)

Acrylamide 40% (Anachem Cat. No. 20-2400-05)	2 ml
1.5M Tris/base pH 8.8 (Promega Cat. No. H5131)	2.51 ml
SDS 20% w/v (Sigma Cat. No. L-3771)	50µl
Ammonium persulphate (AMPS) 10% w/v (Sigma Cat. No. A9164)	100µl
Temed (Promega Cat. No. V3161)	7.5µl
Made up with H_2O.	5.33 ml

Stacking gel (4%) (3.3mls)

Acrylamide 40% (Anachem Cat. No. 20-2400-05)	0.33 ml
1.0M Tris/base pH 6.8 (Promega Cat. No. H5131)	0.42 ml
SDS 20% w/v (Sigma Cat. No. L-3771)	6.7µl
AMPS 10% w/v (Sigma Cat. No. A9164)	33µl
Temed (Promega Cat. No. V3161)	4µl
Made up with H_2O	2.49 ml

Running Buffer pH 8.3

25mM Tris/base (Promega Cat. No. H5131)	3.06 g
190mM Glycine (Sigma Cat. No. G4392)	14.4.g
0.001% SDS (Sigma Cat. No. L-3771)	1g
Made up to 1000mL H_2O	

Blotting/transfer buffer

25mM Tris/base (Promega Cat. No. H5131)	3.06 g
190mM Glycine (Sigma Cat. No. G4392).	14.4 g
20% Methanol (BDH/Merck Cat. No.101586B)	200 ml
Made up to 1000mL H_2O	

Washing buffer

1x PBS (Oxoid Cat. No. BR14a)/ 0.5% Tween 20 (Sigma Cat No. P1379)

Blocking buffer

5% Non fat dried milk-"Marvel®" in 1x PBS/0.5% Tween 20

Nitrocellulose paper

Nitrocellulose paper-hybond-C super (Amersham Life Sciences RPN 203G)

Whatman paper

Whatman International Ltd, Maidstone, UK (Cat. No. 3017915)

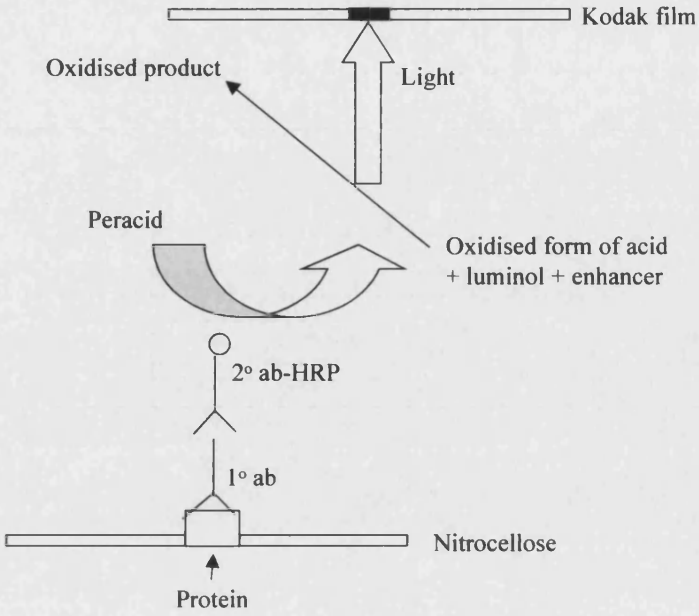
Secondary antibody

Swine Anti rabbit HRP conjugated (Dako)

Amersham ECL detection kit

(Amersham, UK. Cat. No. RPN 2106)
The principle is based on enhanced chemiluminescence, which is achieved by performing the oxidation of luminol by the HRP/hydrogen peroxide system in the presence of chemical enhancers such as phenols. This increases the output of light by approximately 1000 fold.

The detection is summarised in the diagram shown.



Coomassie blue staining of gels

Stain [45% (v/v) methanol, 10% (v/v) acetic acid and 0.1% Coomassie Brilliant blue] (1hr)
De-stain [5% (v/v) methanol and 7.5% (v/v) acetic acid] 3hrs, x 3 changes.
Coomassie Brilliant Blue (Sigma Cat. No. B-0149)

Radiographic film

Kodak X-OMAT AR auto rad film (Amersham Life Sciences, UK Cat. No. V8532665)

XO activity (pterin) assay

Isoxanthopterin

(Sigma. Cat. No. I 7388)
10mM = 9 mg dissolved in 100µl of 1M NaOH in 4900 ml DDW
100µM = 10µl of 10mM stock added to 990 ml DDW

Pterin

(Sigma Cat. No. (P 1132)
10mM = 8.2 mg dissolved in 100µl of 1M NaOH in 4900µl of DDW

Methylene blue

(Sigma Cat. No. MB-1)
10mM = 18.7 mg dissolved in 5 ml DDW

Allopurinol

(Sigma Cat. No. A 8003)
10mM = 6.8 mg dissolved in 100µL of 1 M NaOH in 4900µl DDW

Xanthine oxidase

(Bovine buttermilk) (Biozyme Cat. No. XO2) 1.0-1.5 U/mg protein.

Lucigenin-enhanced chemiluminescence

2mM NADH solution (PBS)
1mM Hypoxanthine (PBS)
2mM Lucigenin solution (PBS)
2mM DTPA solution (PBS)
PBS

ELISA assay

Sample homogenisation buffer

0.1% SDS
0.05% Tween
PBS
1mM PMSF (Sigma. Cat. No. P-7626).
Pepstatin A (1μg/ml) (Sigma. Cat. No. P4265)
Antipain (1μg/ml) (Sigma. Cat. No. A-6191)
Leupeptin (1μg/ml) (Sigma. Cat. No. L-2023)

Carbonate buffer- coating buffer (0.05 M, pH 9.6)

1.59g Na_2CO_3
2.93g $NaHCO_3$
0.20g NaN_3
Made up to 1 litre with DDW

Phosphate buffered saline (PBS)

8.00g NaCl
0.20g KH_2PO_4
2.89g $Na_2HPO_4.12H_2O$
0.20g KCL
Made up to 1 litre with distilled water

Washing buffer (PBS/Tween)

As above + 0.05% Tween (v/v)

Phosphate/citrate buffer

Stock solution A 0.1M citric acid- store at 4°C
Stock solution B 0.2M Na_2HPO_4- store at room temperature
Immediately before use, add 24.3ml A and 25.7ml B to 50ml distilled water in dark bottle.

Peroxidase substrate

Add 57mg OPD (orthophenylenediamine)/100ml phosphate/citrate buffer incorporating 15μl 30% w/w H_2O_2

Stopping solution

4M H_2SO_4

Antibodies

Anti-XO IgM antibody (Neomarkers Mab 3)
Biotinylated anti-XO chicken antibody
Streptavidin-peroxidase conjugate (1μg/ml, Jackson Labs)

Plates

Immulon 1B microtitre plates (Dynatech Lab)

Tissue culture

Hanks balanced salt solution (HBSS)

This contained $Ca^{2+}$and Mg^{2+}, sodium bicarbonate and phenol red and was sterile.
(GibcoBRL, Cat No. 2402 091)

Dulbecco's Modified Eagle Media (DMEM)

With Sodium Pyuruvate, with 1000 mg/ml Glucose, with Pyridoxime
(GibcoBRL, Cat No. 31885-023)

L-Glutamine 200 mm (100x)

(GibcoBRL, Cat No. 25030-024)

Penicillin-Streptomycin (10000 IU/ml- 10000µg/ml)

(GibcoBRL, Cat No. 15140- 122)

Foetal bovine serum (FCS)

Heat inactivated, sterile filtered
(Sigma cell culture, F-9665)

10% FCS/DMEM

450 ml DMEM
50 ml FCS
5 ml L – Glutamine
5 ml Penicillin-streptomycin
All FCS, L- glutamine and penicillin-streptomycin was filtered sterilised through a 0.2µm filter (Triple Red Laboratory technology; Part No. 1520012)

Serum free media

DMEM without phenol red (with Sodium Pyuruvate, with 1000 mg/ml Glucose, with Pyridoxime) (GibcoBRL, Cat No 11880-028)
Bovine serum albumin (BSA) (0.1mg/ml final)
Ascorbate (100mM final)
Transferrin (5µg/ml final)
Insulin (1Mm final)

Trypsin-EDTA solution (10x)

(Sigma cell culture Cat No. T-4174)
5 ml stock added to 45 ml Dulbecco's Phosphate buffered saline (D-PBS). Filtered sterilised through a 0.2µm filter (Triple Red Laboratory technology; Part No. 1520012)

Dulbecco's Phosphate Buffered Saline (D-PBS)

W/o Ca^{2+} & Mg^{2} (Gibco Cat. No.14190-080)

Lab-tek II chamber slide w/cover

8 well (Nalge Nunc International)

Tissue culture equipment (Orange scientific)

Tissue culture test plates	6 wells
Tissue culture flasks (screw cap with filters)	$25cm^2$
Tissue culture flasks (screw cap with filters)	$75cm^2$

Immunocytochemistry

Blocking buffer

5% Non fat dried milk-"Marvel®" in 1x PBS/0.5% Tween 20

Antibodies- XOR

Polyclonal rabbit anti-XOR (Chemicon AB 1242)
Biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Vectastatin® ABC-AP reagent, No AK –5001, Peterborough UK)

Anti-α-actin antibody

Monoclonal anti-α- smooth muscle actin alkaline phosphatase conjugate clone 1A4.
IgG fraction of Mouse ascites fluid (Sigma, product no A5691)
Alkaline phosphatase-conjugated avidin biotin complex (ABC) (Vector Laboratories, Vectastatin® ABC-AP reagent, No AK –5001, Peterborough UK)
Naphthol AS -MX phosphate/Fast red TR Fast™ (Sigma. Cat. No. F-4523)

Cell counting

Bright line hemocytometer (Z35, 962-9) (Sigma)

Cell count and viability assay

1. 100µl aliquots of cell solutions were mixed with equal volumes of 0.4% Trypan blue solution (SIGMA, UK) and allowed to stand for 5 minutes at room temperature.
2. A 10µl aliquot of this solution was added to haemocytometer by capillary action. Cells were viewed by low power transmitted light microscopy and cells within the central $0.1mm^3$ section of the haemocytometer were counted.
3. Total cell counts were calculated by counting all the cells, blue and clear, in the central area of the two counting chambers and dividing by two and then multiplying by $1x10^4$. This figure represents the original cell number before dilution and expressed as cells per ml.
4. Viable counts were calculated by repeating the above procedure by counting only the unstained cells.
5. The percentage viable count was calculated by dividing the clear cell count by the total cell count and multiplying by 100.

RNA/protein extraction

RNA STAT 60™

Total RNA/mRNA isolation reagent (ams Biotechnolgy (Europe) Ltd CS-110

Equipment/reagents

0.2 ml Micro-tube (Certified DNAse, RNAse free) (ABgene Cat no 0620)
DPEC (diethyl pyrocarbonate) (Sigma D5758)
Centrifuge Biofuge fresco (Heraeus instruments)
Chloroform (BDH 277105X)
Isopropanol (Sigma I-0398)
Ethanol (BDH 28304)
Distilled water RNAse, DNAse free (0.1 micron filtered)(GibcoBRL Life technologies Cat no; 1094595)
Guanidine hydrochloride (Promega H5381)
SDS (sodium dodecylsulfate) (Sigma L-3771)

RNA quantification

Quartz cuvette 200µl
UV Biotech photometer (Jencons)
260nm and 280 nm filters

Reverse transcription- Reverse-iT™ First Strand Synthesis Kit (ABgene 0789)

Components used;
Anchored oligo-dT (500ng/µl)
5x first strand synthesis buffer
d NTP mix (5mM each)
Reverse-iT Blend (RTase blend and RNase inhibitor)

PCR Amplification- 2x ReddyMix™ PCR Master Mix (3.0 mM $MgCl_2$) (ABgene 0627-DC-LD)

25µl reaction volume contains 12.5µl of ReddyMix
1.25 units *Taq* DNA Polymerase
75 mM Tris-HCL (pH 8.8 at 25°C)
20 mM $(NH_4)_2SO_4$
3.0 mM $MgCl_2$
0.01% (v/v) Tween 20
0.2 mM each of dATP, dCTP, dGTP, dTTP.
Precipitant and red dye for electrophoresis

XOR primer sequences (10 pmoles/µl)

GibcoBRL Life Technologies
Forward (5′ to 3′) AGT ATG TAC ACA CTG CTC CGG
Reverse (5′ to 3′) GCC TCA GCA ACT CTG GGG GAA
Product size 288 base pairs

GAPDH primer sequences (7.5 pmoles/µl)

R+D Systems, Human GAPDH PCR primer pair (RDP-39)
Forward (5′ to 3′) AAA GGG TCA TCA TCT CTG CC
Reverse (5′ to 3′) TGA CAA AGT GGT CGT TGA GG
Product size 576 base pairs

MMP-2 PCR Primer pair (7.5 pmoles/µl)

R+D Systems, Human MMP-2 primer pair (RDP-84-025)
Product size 449 base pairs

MMP-9 PCR Primer pair (7.5 pmoles/µl)

R+D Systems, Human MMP-9 primer pair (RDP-96-025)
Product size 564 base pairs

Agarose

LE, analytical grade (Promega Cat no; V3121)

DNA ladder

100 bp DNA ladder (Promega Cat no; G210A)
Blue/orange 6x loading dye (Promega Cat no; G190A)

Tris base/borate/EDTA TBE

5 x stock solution 1L

Tris base	54g	(Promega	H5131)
Boric acid	27.5g	(Promega	H5001)
0.5M EDTA	20 ml	(Promega	V4231)

Ethidium Bromide

ICN biomedical Inc (Cat no; 806808)

Electrophoresis apparatus

Run One™-Electrophoresis cell and cast (Embi Tec)

Image capture and analysis

Scion Corporation Imaging program (version 4.0.2; Web site: www.scioncorp.com)

MMP ELISA

Human MMP-2 Immunoassay Kit (Chemicon International (ECM 492)

Human MMP-9 Immunoassay Kit (Chemicon International (ECM 494)

1. Pipette 50µl of each Standard Curve Solution or Specimen into the bottom of a test tube.
2. Pipette 300µl Enzyme Labelled Antibody Solution into each specimen or standard-containing tube.
3. Place one anti-MMP-2/MMP-9 coated bead into each tube.
4. Incubate the tubes at 17-27 ^{O}C for 1 hour.
5. Stop the reaction by adding 3.0ml Washing Solution to each tube
6. Aspirate the solution and dispense 3.0ml washing Solution to each tube.
7. Repeat this washing step 3 times.
8. Transfer each washed bead into a clean fresh tube.
9. Pipette 300µl Colouring Solution into each tube.

10. Incubate the tubes at 17-27 ^{O}C for 1 hour.
11. Stop the enzyme reaction by adding 1.5ml of Stop Solution to each tube.
12. Using deionized water as a blank, read the absorbance at 492 nm for the Standard Curve Solutions and Specimens.
13. Plot the net absorbance value for each MMP-2/MMP-9 concentration versus the MMP-2 concentration (from 6.3 to 400ng/ml) or MMP-9 concentration (from 3.1 to 100ng/ml), and using the net absorbance value for a specimen, determine the respective MMP-2/MMP-9 concentration from the standard curve.

Activity assays

Succinyl trialanyl 4-nitroanilide (SAAANA) assay

96 well plate (Orange scientific)
Succinyl trialanyl 4-nitroanilide (SAAANA) (Sigma)
Dimethyl sulphoxide (DMSO) (Sigma)
Porcine pancreatic elastase (PPE) (Sigma)
50mmol/L Tris-hydrochloride buffer containing 10mmol/L Calcium Chloride ph 7.2

Gelatinase Activity Assay Kit (Chemicon international (ECM701)

1. Rehydrate MMP Positive Control.
2. In a 96-well plate (not provided), add 10µl MMP sample/Positive Control or test sample
3. Add 200µl of Biotinylated Gelatinase Substrate.
4. Cover plate and incubate at 37°C for 30 minutes.
5. Rehydrate Biotin-Binding Plate by adding 200 µL of diluted Assay Buffer to each well and let stand at room temperature for 1 minute.
6. Aspirate Assay Buffer from Biotin-Binding Plate and add 100 µL of the MMP/Substrate mixture from step 3 to the Biotin-Binding plate.
7. Incubate at 37°C for 2 hours.
8. Wash each well 5 times with 200 µL of diluted assay buffer.
9. Prepare a 1:3,000 dilution of Streptavidin-Enzyme Conjugate in Assay Buffer (add 4 µL of conjugate to12 ml of Assay Buffer).
10. Add 100 µL of diluted conjugate to each well.
11. Incubate at 37°C for 30 minutes.
12. Wash each well 5 x with 200 µL of diluted Assay Buffer.
13. Add 100 µL of substrate solution.
14. Incubate at room temperature for 20 minutes.
15. Positive control wells should develop a faint colour.
16. Stop reaction by adding 100 µL of Stop Solution to each well.
17. Read O.D. at 450 nm.

Succinylated Elastin assay

Succinic anhydride (Sigma S7626)
Gelatin (Sigma G2500)
Purified human MMP-2 (Gelatinase A) (Chemicon CC071)
Purified human MMP-9 (Gelatinase B) (Chemicon CC079)
p-Aminophenylmercuric acetate (APMA) (Sigma A9563)
2,4,6-trinitrobenzene sulfonic acid (TNBSA) (Sigma)